USER GUIDE

Ion Total RNA-Seq Kit v2

for use with:

Ion Personal Genome Machine® (PGM™) System

Ion Proton™ System

Catalog Number 4475936 and 4479789

Publication Number 4476286

Revision D

For research use only. Not for use in diagnostic procedures.

For Research Use Only. Not for use in diagnostic procedures.

The information in this guide is subject to change without notice.

DISCLAIMERS

LIFE TECHNOLOGIES CORPORATION AND/OR ITS AFFILIATE(S) DISCLAIM ALL WARRANTIES WITH RESPECT TO THIS DOCUMENT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, OR NON-INFRINGEMENT. TO THE EXTENT ALLOWED BY LAW, IN NO EVENT SHALL LIFE TECHNOLOGIES AND/OR ITS AFFILIATE(S) BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING BUT NOT LIMITED TO THE USE THEREOF.

TRADEMARKS

The trademarks mentioned herein are the property of Life Technologies Corporation and/or its affiliate(s) or their respective owners.

Bioanalyzer and Agilent are registered trademarks of Agilent Technologies, Inc. TaqMan is a registered trademark of Roche Molecular Systems, Inc. and is used under permission and license. NanoDrop is a registered trademark of NanoDrop Technologies LLC. SpeedVac is a registered trademark of Thermo Fisher Scientific.

© 2012 **Life Technologies Corporation.** All rights reserved.

Contents

About This Guide 7

Revision history 7
Other Ion library preparation kits and guides 7
Ambion® products and expertise 7

CHAPTER 1 Product Information 9

Purpose of the product 9
Preparing barcoded libraries 9
Kit components and storage 10
Ion Total RNA-Seq Kit v2, 12-Reaction Kit 10
Ion Total RNA-Seq Kit v2, 48-Reaction Kit 11
Materials and equipment required but not provided 12
(*Optional*) Ion Xpress™ RNA-Seq Barcode 01–16 Kit 12
Required for library preparation 12
Materials for whole transcriptome libraries 13
Materials for small RNA libraries 13

CHAPTER 2 Prepare Whole Transcriptome Libraries 15

Workflow 16
Fragment the whole transcriptome RNA 17
Guidelines for RNA sample type and amount 17
ERCC RNA Spike-In Control Mixes 17
Fragment the RNA using RNase III 18
Purify the fragmented RNA 19
Assess the yield and size distribution of the fragmented RNA 21
Typical results of fragmentation of whole transcriptome RNA 22
Construct the whole transcriptome library 24
Hybridize and ligate the RNA 24
Perform reverse transcription (RT) 26
Purify the cDNA 27
Amplify the cDNA 29
Purify the amplified cDNA 30
Assess the yield and size distribution of the amplified DNA 32
Pool barcoded whole transcriptome libraries 33
Determine the library dilution required for template preparation 34
Proceed to template preparation 34

Size distributions and yields 35
Typical size distributions 35
Expected yields 39
Troubleshooting 40
Using a positive control to troubleshoot 40

CHAPTER 3 Prepare Small RNA Libraries 41

Workflow 42
Prepare the starting material 43
Guidelines for obtaining small RNA 43
Assess the amount and quality of small RNA in your total RNA samples 43
Enrich the samples for small RNA 44
Assess the quality and quantity of the small RNA-enriched sample 48
Determine the input amount 48
Construct the small RNA library 49
Hybridize and ligate the RNA 49
Perform reverse transcription 51
Purify and size-select the cDNA 52
Amplify the cDNA 54
Purify and size-select the amplified DNA 56
Assess the yield and size distribution of the amplified DNA 59
Pool barcoded small RNA libraries 60
Determine the library dilution required for template preparation 60
Proceed to template preparation 61
Typical size distribution 61
Plotted size distributions 61
Size distributions compared 64
Troubleshooting 65
Using a positive control to troubleshoot 65

APPENDIX A Supplemental Information 67

Amplified library construction concepts 67
Hybridization and ligation to the Adaptor Mix 67
Reverse transcription and size-selection 67
Using 2100 expert software to assess whole transcriptome libraries 68
Perform a smear analysis 68
Analyze multiple peaks as one peak 69
Using 2100 expert software to assess small RNA libraries 72
Review the median size 72
Perform a smear analysis 72
Determine the % miRNA library 74

APPENDIX B Safety 75

Chemical safety 76
Biological hazard safety 77

Documentation and Support 79

Obtaining SDSs 79
Obtaining Certificates of Analysis 79
Obtaining support 79
Ion contact information 80
Limited Product Warranty 80

About This Guide

- Revision history 7
- Other Ion library preparation kits and guides 7
- Ambion® products and expertise 7

IMPORTANT! Before using this product, read and understand the information in Appendix B, "Safety" on page 75 in this document.

Revision history

Revision	Date	Description of change
D	17 December 2012	• Combined the whole transcriptome protocol for standard and low RNA inputs. • Added support for the Ion Proton™ System. • Placed optional materials in Chapter 1, "Product Information" and removed index.
C	22 August 2012	• Added the new 48-reaction kit, the Ion Total RNA-Seq Kit v2, 48-Reaction Kit (Cat. no. 4479789).

Other Ion library preparation kits and guides

Other library preparation kits and protocols are available. For guides and protocols, visit the Ion community at **http://ioncommunity.iontorrent.com/** and follow the links under **Protocols** > **Construct Library** > **Construct Library User Guides and Quick Reference**.

Ambion® products and expertise

Backed by more than 20 years of experience, Ambion® products provide innovative solutions for specialized RNA applications including RNA Sequencing. Life Technologies has combined Ambion® RNA isolation and gene expression analysis experience with the speed and simplicity of the Ion Torrent PGM™ platform to optimize each module in the Ion Total RNA-Seq Kit v2. Therefore, these products provide the highest possible performance in the RNA-Seq workflow.

1 Product Information

- Purpose of the product 9
  - Preparing barcoded libraries 9
- Kit components and storage 10
  - Ion Total RNA-Seq Kit v2, 12-Reaction Kit 10
  - Ion Total RNA-Seq Kit v2, 48-Reaction Kit 11
- Materials and equipment required but not provided 12
  - (Optional) Ion Xpress™ RNA-Seq Barcode 01–16 Kit. 12
  - Required for library preparation 12
  - Materials for whole transcriptome libraries 13
  - Materials for small RNA libraries 13

Purpose of the product

Use the Ion Total RNA-Seq Kit v2.0 (Cat. no. 4475936 and 4479789) to convert RNA transcripts expressed in a cell or tissue into representative cDNA libraries for strand-specific RNA sequencing on the Ion Torrent Personal Genome Machine® (PGM™) System and the Ion Proton™ System. This user guide supports library preparation for up to 200-base-read sequencing:

- For whole transcriptome libraries, follow the procedures in Chapter 2 on page 15.
- For small RNA libraries, follow the procedures in Chapter 3 on page 41.

Preparing barcoded libraries

The Ion Total RNA-Seq Kit v2.0 supports barcoded library preparation to enable sequencing of multiple samples in a single, multiplexed sequencing run. To prepare barcoded libraries, replace the adaptors in the kit with adaptors from the Ion Xpress™ RNA-Seq Barcode 01–16 Kit (Cat. no. 4475485).

Kit components and storage

Ion Total RNA-Seq Kit v2, 12-Reaction Kit

Sufficient reagents are supplied in the Ion Total RNA-Seq Kit v2, 12-Reaction Kit (Cat. no. 4475936) to prepare cDNA libraries from 12 samples for sequencing analysis with the Ion PGM™ System.

Box	Components†	Cap Color	Quantity	Volume	Storage
Ion RNA-Seq Core Kit v2, 12-Reaction Kit (Part no. 4474906)	Nuclease-Free Water	Clear	2	1.75 mL	15°C to 30°C (room temperature)
	10X RNase III Reaction Buffer	Red	1	20 µL	–30°C to –10°C
	RNase III	Red	1	20 µL	
	Hybridization Solution	Green	1	40 µL	
	2X Ligation Buffer	Green	1	150 µL	
	Ligation Enzyme Mix	Green	1	30 µL	
	10X RT Buffer	Yellow	1	56 µL	
	2.5 mM dNTP Mix	White	1	30 µL	
	10X SuperScript® III Enzyme Mix	Yellow	1	56 µL	
	Platinum® PCR SuperMix High Fidelity	Blue	1	900 µL	
	WT Control RNA (1 µg/µL HeLa total RNA)	Clear	1	50 µL	
	Small RNA Control (1 µg/µL human placenta total RNA)	Purple	1	10 µL	
Ion RNA-Seq Primer Set v2, 12-Reaction Kit (Part no. 4474810)	Ion Adaptor Mix v2	Green	1 tube	30 µL	–30°C to –10°C
	Ion RT Primer v2	Yellow	1 tube	104 µL	
	Ion 5′ PCR Primer v2	White	1 tube	20 µL	
	Ion 3′ PCR Primer v2	Blue	1 tube	20 µL	
Magnetic Bead Cleanup Module (Part no. 4475486)	Processing Plate	—	1	—	15°C to 30°C (room temperature)
	Binding Solution Concentrate	—	1 bottle	11 mL	
	Wash Solution Concentrate	—	1 bottle	11 mL	
	Nucleic Acid Binding Beads	Clear	1 tube	0.7 mL	2°C to 8°C **IMPORTANT!** Do not freeze.

† Life Technologies has validated this protocol using this specific material. Substitution may adversely affect performance.

Ion Total RNA-Seq Kit v2, 48-Reaction Kit

Sufficient reagents are supplied in the Ion Total RNA-Seq Kit v2, 48-Reaction Kit (Cat. no. 4479789) to prepare cDNA libraries from 48 samples for sequencing analysis with the Ion PGM™ System and Ion Proton™ System.

Box	Components†	Cap Color	Quantity	Volume	Storage
Ion RNA-Seq Core Kit v2, 48-Reaction Kit (Part no. 4479687)	10X RNase III Reaction Buffer	Red	1	60 µL	–30°C to –10°C
	RNase III	Red	1	60 µL	
	Hybridization Solution	Green	1	170 µL	
	2X Ligation Buffer	Green	1	650 µL	
	Ligation Enzyme Mix	Green	1	110 µL	
	10X RT Buffer	Yellow	1	224 µL	
	2.5 mM dNTP Mix	White	1	120 µL	
	10X SuperScript® III Enzyme Mix	Yellow	1	224 µL	
	Platinum® PCR SuperMix High Fidelity	Blue	2	1800 µL	
	WT Control RNA (1 µg/µL HeLa total RNA)	Clear	1	50 µL	
	Small RNA Control (1 µg/µL human placenta total RNA)	Purple	1	10 µL	
Ion RNA-Seq Primer Set v2, 48-Reaction Kit (Part no. 4475482)	Ion Adaptor Mix v2	Green	1 tube	120 µL	–30°C to –10°C
	Ion RT Primer v2	Yellow	1 tube	416 µL	
	Ion 5′ PCR Primer v2	White	1 tube	80 µL	
	Ion 3′ PCR Primer v2	Blue	1 tube	80 µL	
Ion RNA-Seq Core Kit v2, 48-Reaction Kit (Part no. 4479687)	10X RNase III Reaction Buffer	Red	1	60 µL	–30°C to –10°C
Magnetic Bead Cleanup Module (Part no. 4475486)	Processing Plate	—	1	—	15°C to 30°C (room temperature)
	Binding Solution Concentrate	—	1 bottle	11 mL	
	Wash Solution Concentrate	—	1 bottle	11 mL	
	Nucleic Acid Binding Beads	Clear	1 tube	0.7 mL	2°C to 8°C **IMPORTANT!** Do not freeze.
	Nuclease-Free Water	—	1 bottle	10 mL	15°C to 30°C (room temperature)

† Life Technologies has validated this protocol using this specific material. Substitution may adversely affect performance.

Materials and equipment required but not provided

For the Safety Data Sheet (SDS) of any chemical not distributed by Life Technologies, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

(*Optional*) Ion Xpress™ RNA-Seq Barcode 01–16 Kit

Sufficient PCR primers are supplied in the Ion Xpress™ RNA-Seq Barcode 01–16 Kit (Cat. no. 4475485) to prepare barcoded cDNA libraries from 12 samples.

Components	Cap color	Quantity	Volume	Storage
Ion Xpress™ RNA BC 01–BC 16	White	1 each	12 µL	–30°C to –10°C
Ion Xpress™ RNA 3′ Barcode Primer	Blue	1 each	192 µL	

Required for library preparation

Item	Source/Cat. No.
Thermal cycler with heated lid, capable of holding 0.2-mL tubes: • Veriti® 96-Well Thermal Cycler • GeneAmp® PCR System 9700	Life Technologies • 4375786 • Various
Agilent® 2100 Bioanalzyer® instrument	Agilent G2938A
NanoDrop® Spectrophotometer	Thermo Scientific
Qubit® 2.0 Fluorometer	Life Technologies Q32866
Centrifugal vacuum concentrator (for example, SpeedVac)	Major Laboratory Supplier (MLS)
Microcentrifuge	MLS
Pipettors, positive-displacement or air-displacement	MLS
Magnetic stand – one of the following: • Magnetic Stand-96 • 96 well Magnetic-Ring Stand	Life Technologies: • AM10027 • AM10050
(*Optional*) Multi-channel pipettor	MLS
Agilent® DNA 1000 Kit	Agilent 5067-1504
Agilent® High Sensitivity DNA Kit	Agilent 5067-4626
Ethanol, 100%, ACS reagent grade or equivalent	MLS
(*Optional*) Qubit® dsDNA HS Assay Kit, 100 assays	Life Technologies Q32851
8-strip PCR Tubes & Caps, RNase-free, 0.2-mL	Life Technologies AM12230
Non-Stick RNase-Free Microfuge Tubes (0.5-mL), 500	Life Technologies AM12350
Non-Stick RNase-Free Microfuge Tubes (1.5-mL), 250	Life Technologies AM12450
Pipette tips, RNase-free	MLS

Item	Source/Cat. No.
(*Optional*) 1.2-mL 96-well plates	Thermal Fisher AB-1127
(*Optional*) FirstChoice® Total RNA	Life Technologies (various cat. no.)

Materials for whole transcriptome libraries

Item	Source/Cat. No.
Qubit® RNA Assay Kit, 100 assays	Life Technologies Q32852
Agilent® RNA 6000 Pico Kit	Agilent 5067-1513
(*Optional*) ERCC RNA Spike-In Control Mixes **Note:** ERCC controls are highly recommended.	Life Technologies 4456739 and 4456740
(*Optional*) Dynabeads® mRNA DIRECT™ Micro Kit	Life Technologies 61021
(*Optional*) MicroPoly(A)Purist™ Kit	Life Technologies AM1919
(*Optional*) TaqMan® Gene Expression Assays for ERCC Targets	Life Technologies (Various cat. no.)
(*Optional*) TaqMan® Gene Expression Master Mix	Life Technologies 4369016 and 4369510
(*Optional*) RiboMinus™ Eukaryote System v2	Life Technologies A15027
(*Optional*) RiboMinus™ Plant Kit for RNA-Seq	Life Technologies A1083808

Materials for small RNA libraries

Item	Source
Agilent® RNA 6000 Nano Kit	Agilent 5067-1511
Agilent® Small RNA Kit	Agilent 5067-1548
(*Optional*) *mir*Vana™ miRNA Isolation Kit, 40 purifications	Life Technologies AM1560
(*Optional*) *mir*Vana™ PARIS™, 40 purification systems	Life Technologies AM1556
(*Optional*) PureLink® miRNA Isolation Kit, 25 preps	Life Technologies K1570-01

2 Prepare Whole Transcriptome Libraries

- Workflow 16
- Fragment the whole transcriptome RNA 17
  - Guidelines for RNA sample type and amount 17
  - ERCC RNA Spike-In Control Mixes 17
  - Fragment the RNA using RNase III 18
  - Purify the fragmented RNA 19
  - Assess the yield and size distribution of the fragmented RNA 21
  - Typical results of fragmentation of whole transcriptome RNA 22
- Construct the whole transcriptome library 24
  - Hybridize and ligate the RNA 24
  - Perform reverse transcription (RT) 26
  - Purify the cDNA 27
  - Amplify the cDNA 29
  - Purify the amplified cDNA 30
  - Assess the yield and size distribution of the amplified DNA 32
- Pool barcoded whole transcriptome libraries 33
- Determine the library dilution required for template preparation 34
- Proceed to template preparation 34
- Size distributions and yields 35
  - Typical size distributions 35
  - Expected yields 39
- Troubleshooting 40
  - Using a positive control to troubleshoot 40

Workflow

Fragment the whole transcriptome RNA

Start with RNA with ERCC RNA Spike-In Control Mix (page 17)

▼

Fragment the RNA using RNase III (page 18)

▼

Purify the fragmented RNA (page 19)

Fragmented RNA

▼

Assess the yield and size distribution of the fragmented RNA (page 21)

▼

Construct the whole transcriptome library

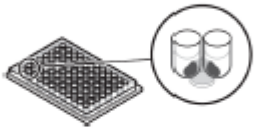
Hybridize and ligate the RNA (page 24)

▼

Perform reverse transcription (RT) (page 26)

▼

Purify the cDNA (page 27)



▼

Amplify the cDNA (page 29)

▼

Purify the amplified cDNA (page 30)

▼

Assess the yield and size distribution of the amplified DNA (page 32)

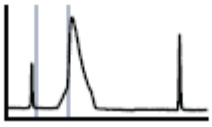
▼

Pool barcoded whole transcriptome libraries (page 33)

▼

Determine the library dilution required for template preparation (page 34)

▼



Prepare templated Ion Sphere™ Particles for sequencing on the Ion PGM™ System

Refer to the specific user guide for an Ion template preparation kit

Fragment the whole transcriptome RNA

Fragmenting the whole transcriptome RNA involves the following procedures:

Fragment the RNA using RNase III (page 18)

⬇

Purify the fragmented RNA (page 19)

⬇

Assess the yield and size distribution of the fragmented RNA (page 21)

Guidelines for RNA sample type and amount

We strongly recommend using 1–500 ng of poly(A) RNA, or 10–500 ng of rRNA-depleted total RNA. You may also use high-quality total RNA.

Guidelines for using poly(A) RNA

To prepare poly(A) RNA from:

- 100 ng–50 µg total RNA, we recommend using the Dynabeads® mRNA DIRECT™ Micro Kit (Cat. no. 61021). Refer to the *Dynabeads® mRNA DIRECT™ Micro Kit User Guide*.
- 50–400 µg total RNA, we recommend performing two rounds of oligo(dT) selection of the poly(A) RNA using the MicroPoly(A)Purist™ Kit (Cat. no. AM1919). Also, confirm the absence of 18S and 28S rRNA; for example, check the profile of the poly(A) RNA on an Agilent® 2100 Bioanalyzer® instrument.

Guidelines for using rRNA-depleted total RNA

To prepare rRNA-depleted total RNA from:

- 1–5 µg total RNA, we recommend that you remove rRNA using the RiboMinus™ Eukaryote System v2 (Cat. no. A15026).
- 100 ng–1 µg total RNA, we recommend that you remove rRNA using the Low Input RiboMinus™ Eukaryote System v2 (Cat. no. A15027).

Guidelines for using total RNA

Best results are obtained when using RNA with an RNA integrity number (RIN) greater than 7. FirstChoice® Total RNA provides high-quality, intact RNA isolated from a variety of sources. Quantitate the amount of RNA in the sample using the NanoDrop® Spectrophotometer.

ERCC RNA Spike-In Control Mixes

We strongly recommend that you add ERCC RNA Spike-In Control Mixes to the input total RNA before RNA depletion or poly(A) selection for whole transcriptome library generation.

The ERCC RNA Spike-In Control Mixes provide a set of external RNA controls that enable performance assessment of a variety of technology platforms used for gene expression experiments. Add one Spike-In Control Mix to each RNA sample, and run these samples on your platform; compare the Spike-In Control Mix data to known

Spike-In Control Mix concentrations and ratios to assess the dynamic range, lower limit of detection, and fold-change response of your platform. The following table provides guidelines for how much Spike-In Control Mix to add to the input RNA for whole transcriptome library preparation. For detailed information, refer to the *ERCC RNA Spike-In Control Mixes User Guide*:

Amount of Sample RNA	Volume of Spike-In Mix 1 or Mix 2 (dilution)†	
	Total RNA	Poly(A) RNA
1 ng	—	1 µL (1:1000)
5 ng	—	5 µL (1:1000)
10 ng	—	1 µL (1:100)
50 ng	—	5 µL (1:100)
100 ng	2 µL (1:1000)	1 µL (1:10)
500 ng	1 µL (1:100)	5 µL (1:10)
1000 ng	2 µL (1:100)	—
5000 ng	1 µL (1:10)	—

† ERCC RNA Spike-In Mix 1, ExFold Spike-In Mix 1, or ExFold Spike-In Mix 2.

ERCC_Analysis Plugin

The ERCC_Analysis plugin is intended to help with ERCC RNA Spike-in Controls. It enables you to quickly determine whether or not the ERCC results indicate a problem with library preparation or the PGM run.

For more information about the ERCC_Analysis Plugin, refer to the *ERCC_Analysis Plugin User Bulletin* (Pub. no. 4479068).

Fragment the RNA using RNase III

Use components from the Ion Total RNA-Seq Kit v2:

- Nuclease-Free Water
- 10X RNase III Reaction Buffer
- RNase III

1. On ice, assemble a reaction for each RNA sample in a 0.2-mL PCR tube:

Component (add in the order shown)	Volume for One Reaction
1. RNA sample and Nuclease-Free Water: • Poly(A) RNA: 1–500 ng • rRNA-depleted total RNA: 10–500 ng • WT Control RNA: 500 ng	8–10 µL
2. 10X RNase III Reaction Buffer	1 µL
3. RNase III	1 µL
Total volume	**10–12 µL**

IMPORTANT! To reduce fragmentation variability, accurately pipet 1 µL of 10X RNase III Reaction Buffer and 1 µL of RNase III to each sample. Do not make a master mix that contains only 10X RNase III Reaction Buffer and RNase III.

2. Flick the tube or pipet up and down 5 times to mix, then centrifuge briefly to collect the liquid in the bottom of the tube.
3. Incubate the reaction in a thermal cycler at 37°C according to library and input quantity:

RNA Type	Amount	Reaction Time
Poly(A) RNA	1 to <100 ng	3 min
	100–500 ng	10 min
rRNA-depleted RNA	10 to <100 ng	3 min
	100–500 ng	10 min
Total RNA	500 ng	10 min

4. *Immediately* after the incubation, add 20 µL of Nuclease-Free Water, then place the fragmented RNA on ice.

IMPORTANT! Proceed immediately to "Purify the fragmented RNA", or leave the fragmented RNA on ice for less than 1 hour.

Purify the fragmented RNA

Required materials from the Magnetic Bead Cleanup Module

- Wash Solution Concentrate
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate
- Nuclease-Free Water

Other materials and equipment

- 100% ethanol or 200 proof (absolute) ethanol, ACS-grade or higher quality
- Magnetic stand for 96-well plates (Life Technologies, Catalog no. AM10027 or AM10050)
- 37°C heat block or water bath
- (*Optional*) MicroAmp® Clear Adhesive Film (Life Technologies, Cat. no. 4306311)

Before you begin

- Add 44 mL of 100% ethanol to the bottle of Wash Solution Concentrate and mix well. Mark the label on the bottle to indicate that you added ethanol. Store the solution at room temperature (15°C to 30°C).
- If you see a white precipitate in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Nuclease-Free Water at 37°C for ≥5 minutes.

Note: To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp® Clear Adhesive Film (Life Technologies, Cat. no. 4306311). You can also skip a row between sample rows.

IMPORTANT! Accurate pipetting of bead cleanup reagents is critical for best results.

1. Prepare beads for each sample:
    a. Gently vortex the Nucleic Acid Binding Beads tube to completely resuspend the magnetic beads.
    b. Add 5 µL beads to wells on the Processing Plate.
    c. Add 90 µL Binding Solution Concentrate to each well, then mix the Concentrate and beads by pipetting up and down 10 times.
2. Bind the fragment RNA products to the beads:
    a. Transfer each 30-µL fragment RNA reaction to a bead-containing well on the Processing Plate.
    b. Set a P200 pipettor at 150 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the new 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times.
    c. Without changing tips, add 150 µL of 100% ethanol to each well.

    IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 2b–2c for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

    d. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

    Note: The color of the mixture should be homogeneous after mixing.

    e. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.
3. Remove the supernatant from the beads:
    a. Place the Processing Plate on a magnetic stand for 5–6 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
    b. Leave the Processing Plate on the magnetic stand, then aspirate and discard the supernatant from the plate.

    IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 microliters of supernatant behind.

4. Wash the beads with Wash Solution Concentrate with ethanol:
    a. Leave the Processing Plate on the magnetic stand.
    b. Add 150 µL of Wash Solution Concentrate with ethanol to each sample.
    c. Incubate the samples at room temperature for 30 seconds.
5. Remove the supernatant from the beads:
    a. Aspirate and discard the supernatant from the plate.
    b. Use a P10 or P20 pipettor to remove any residual liquid.

    IMPORTANT! Do not disturb the separated magnetic beads. Remove all of the Wash Solution Concentrate from each well.

c. Air-dry the beads at room temperature to remove all traces of ethanol by leaving the Processing Plate on the magnetic stand for 1–2 minutes.

IMPORTANT! Do not overdry the beads (overdried beads appear cracked). Overdrying significantly decreases elution efficiency.

6. Elute the fragmented RNA from the beads:
   a. Remove the Processing Plate from the magnetic stand.
   b. Add 12 µL of pre-warmed (37°C) Nuclease-Free Water to each sample, then mix the Nuclease-Free Water and beads by pipetting up and down 10 times.
   c. Incubate at room temperature for 1 minute.
   d. Place the Processing Plate on the magnetic stand for 1 minute to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
   e. For each sample, collect the eluant.

Assess the yield and size distribution of the fragmented RNA

Use the Qubit® RNA Assay Kit with the Qubit® Fluorometer and the Agilent® RNA 6000 Pico Kit with the Agilent® 2100 Bioanalyzer® instrument.

Note: You can use a NanoDrop® Spectrophotometer in place of the Qubit® RNA Assay Kit and Qubit® Fluorometer. For increased accuracy, quantitate the RNA concentration using the Qubit® RNA Assay Kit with the Qubit® Fluorometer.

Note: Assessing the yield and size is not recommended for poly(A) fragmented RNA samples from <5 ng poly(A) RNA due to low input amount.

1. Quantitate the yield of the fragmented RNA using the Qubit® RNA Assay Kit with the Qubit® Fluorometer.

   Refer to the *Qubit® RNA Assay Kit Protocol* (Pub. no. MAN0002327) or the *Qubit® Fluorometer Instruction Manual* (Pub. no. MAN0002328) for instructions.

2. Assess the size distribution of the fragmented RNA:
   a. If necessary, dilute 1 µL of the sample to 50-5000 pg/µL with Nuclease-Free Water.
   b. Run the diluted sample on an Agilent® 2100 Bioanalyzer® instrument with the RNA 6000 Pico Kit. Follow the manufacturer's instructions for performing the assay.
   c. Using the 2100 expert software, review the size distribution.

      The fragmentation procedure should produce a distribution of RNA fragment sizes from 35 nt to several hundred or a few thousand nt, depending on your sample type. The average size should be 100–200 nt. See the figures starting on page 22.

      Note: For instructions on how to review the size distribution, refer to the *Agilent® 2100 Bioanalyzer® Expert User's Guide* (Pub. no. G2946-9000).

      If the profile for the fragmented RNA does not meet the typical results, see "Troubleshooting" on page 40 for guidance.

3. Proceed according to the amount of fragmented RNA you have in 3 µL:

Amount of Fragmented RNA in 3 µL	Instructions
• ≥50 ng of poly(A) RNA • ≥100 ng of rRNA-depleted total RNA • ≥100 ng of WT Control RNA	Proceed to "Construct the whole transcriptome library" on page 24. Store the remaining RNA at –86°C to –68°C.
• <50 ng of poly(A) RNA • <100 of ng rRNA-depleted total RNA	1. Dry all of the RNA completely in a centrifugal vacuum concentrator at low or medium heat (≤40°C); this should take 10–20 minutes. 2. Resuspend in 3 µL of Nuclease-Free Water, then proceed to "Construct the whole transcriptome library" on page 24.

Typical results of fragmentation of whole transcriptome RNA

The figures in this section show profiles from an Agilent® 2100 Bioanalyzer® instrument after RNase III fragmentation and cleanup. Figure 1 shows results with HeLa poly(A) RNA. Figure 2 shows results with HeLa rRNA-depleted total RNA.

Figure 1 Size distribution of fragmented HeLa poly(A) RNA

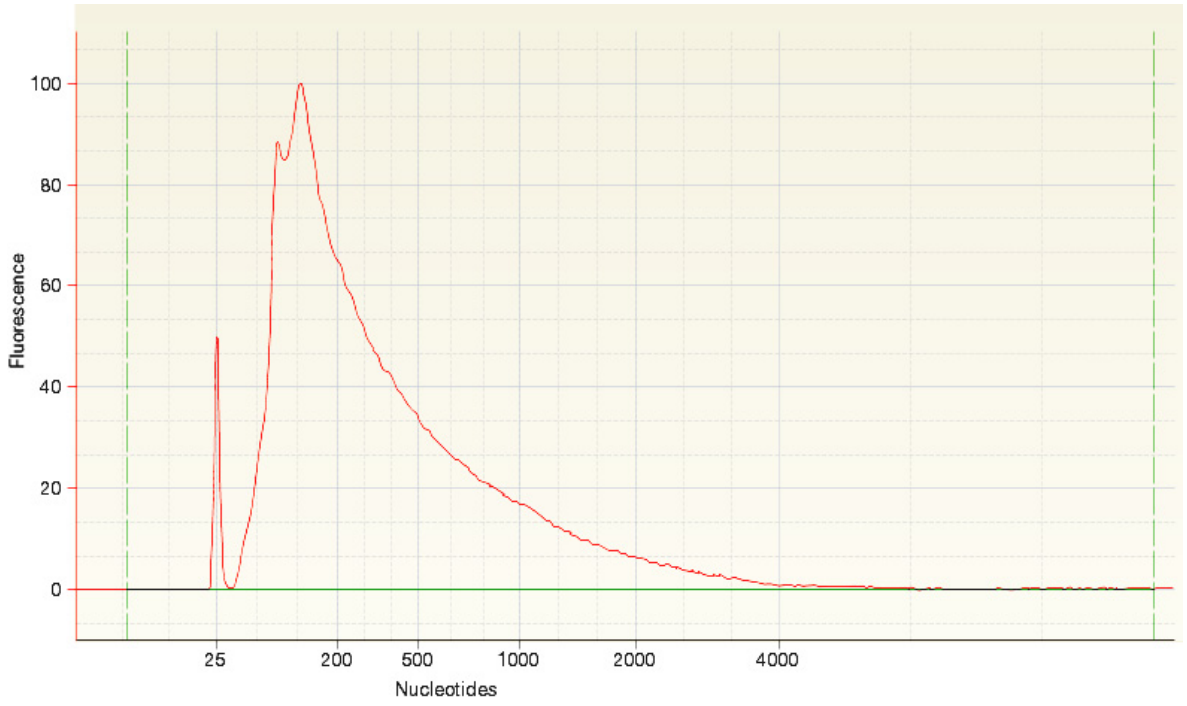
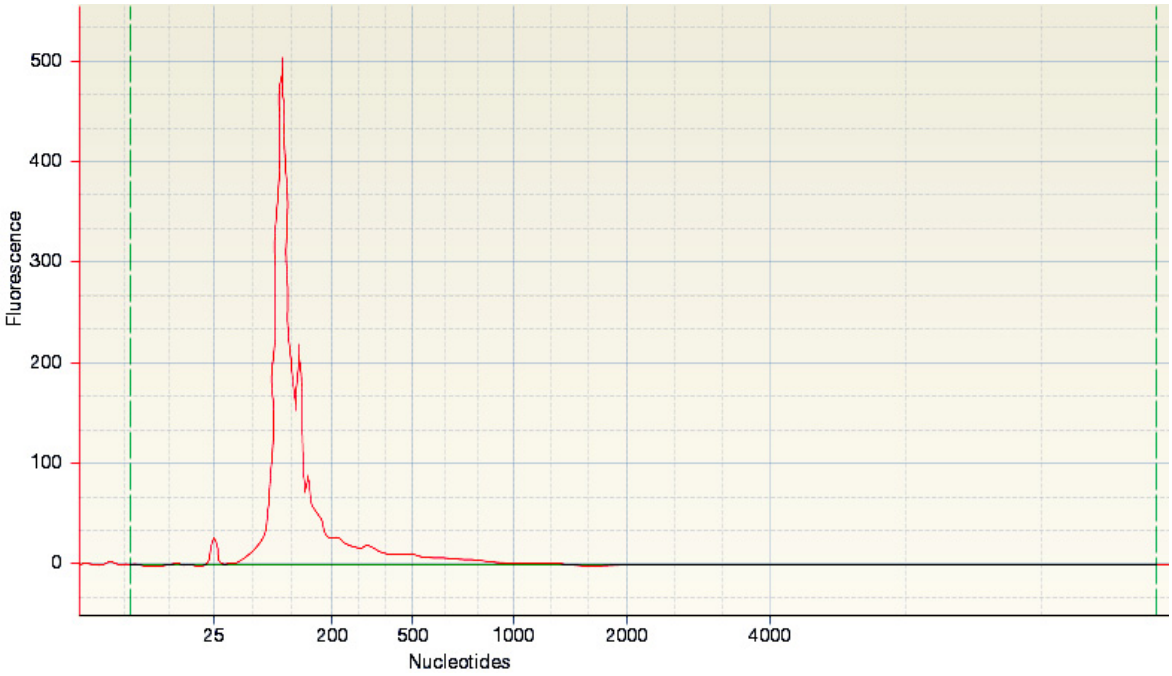


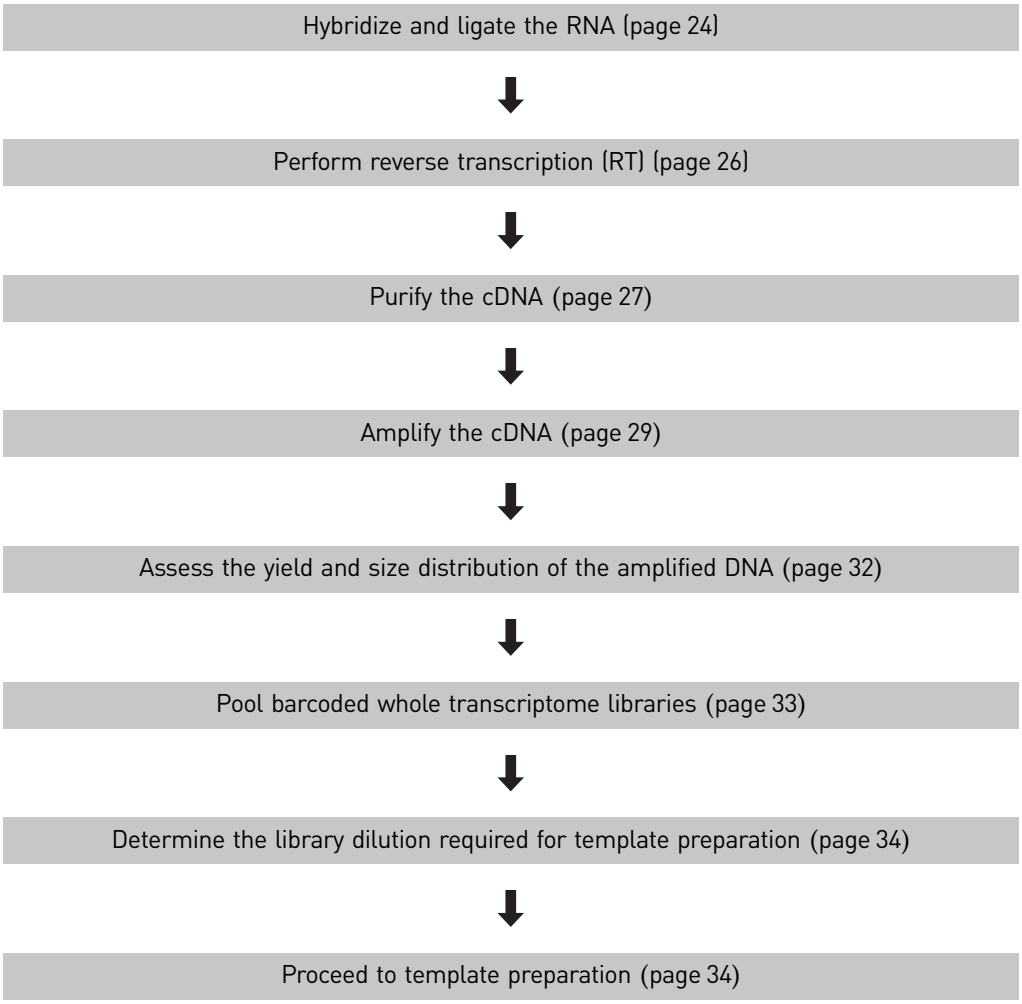
Figure 2 Size distribution of fragmented HeLa rRNA-depleted total RNA



Construct the whole transcriptome library

IMPORTANT! The Ion Adaptor Mix v2, Ion RT Primer v2, and Ion PCR primers are unique to the Ion Total-RNA Seq Kit v2. Do *not* use the reagents from the Ion Total-RNA Seq Kit (first version) to prepare libraries with this user guide.

Constructing the whole transcriptome library involves the following procedures:


Hybridize and ligate the RNA (page 24)
Perform reverse transcription (RT) (page 26)
Purify the cDNA (page 27)
Amplify the cDNA (page 29)
Assess the yield and size distribution of the amplified DNA (page 32)
Pool barcoded whole transcriptome libraries (page 33)
Determine the library dilution required for template preparation (page 34)
Proceed to template preparation (page 34)

Hybridize and ligate the RNA

Use components from the Ion Total RNA-Seq Kit v2:

- Ion Adaptor Mix v2
- Hybridization Solution
- Nuclease-Free Water
- 2X Ligation Buffer
- Ligation Enzyme Mix

1. On ice, prepare the hybridization master mix:

Component	Volume for One Reaction†
Ion Adaptor Mix v2	2 µL
Hybridization Solution	3 µL
Total volume	**5 µL**

† Include 5–10% excess volume to compensate for pipetting error when preparing the master mix.

2. Add 5 µL of hybridization master mix to 3 µL of fragmented RNA sample:
   - Fragmented poly(A) RNA: up to 50 ng
   - Fragmented rRNA-depleted total RNA: up to 100 ng

   Note: If <50 ng of fragmented poly(A) RNA or <100 ng rRNA-depleted total RNA is recovered after fragmentation, we recommend using all fragmented RNA for ligation.
3. Slowly pipet the solution up and down 10 times to mix, then centrifuge briefly.
4. Run the hybridization reaction in a thermal cycler:

Temperature	Time
65°C	10 min
30°C	5 min

5. On ice, add the RNA ligation reagents to the 8-µL hybridization reactions:
   a. Combine:

Component	Volume for One Reaction†
Hybridization reaction	8 µL
2X Ligation Buffer	10 µL
Ligation Enzyme Mix	2 µL
Total volume	**20 µL**

† Include 5–10% excess volume to compensate for pipetting error when preparing the master mix.

IMPORTANT! If the 2X Ligation Buffer contains a white precipitate, warm the tube at 37°C for 2–5 minutes or until the precipitate is dissolved. 2X Ligation Buffer is very viscous; pipet slowly to dispense it accurately.

   b. Flick the tube or slowly pipet the solution up and down 5 times to mix well, then centrifuge briefly to collect the liquid in the bottom of the tube.

6. Incubate the 20-µL ligation reactions in a thermal cycler at 30°C according to input type and amount:

RNA Type	Amount into Fragmentation	Reaction Time
Poly(A) RNA	1–5 ng	1 hour
	>5 ng	30 min
rRNA-depleted RNA	10–100 ng	1 hour
	>100 ng	30 min

IMPORTANT! Set the temperature of the thermal cycler lid to match the block temperature; turn OFF the heated lid; or leave the thermal cycler open during the incubation.

Perform reverse transcription (RT)

Use components from the Ion Total RNA-Seq Kit v2:

- Nuclease-Free Water
- 10X RT Buffer
- 2.5 mM dNTP Mix
- Ion RT Primer v2
- 10X SuperScript® III Enzyme Mix

1. On ice, prepare the RT master mix:

Component	Volume for One Reaction†
Nuclease-Free Water	2 µL
10X RT Buffer	4 µL
2.5 mM dNTP Mix	2 µL
Ion RT Primer v2	8 µL
Total volume	**16 µL**

† Include 5–10% excess volume to compensate for pipetting error when preparing the master mix.

2. Incubate the RT master mix with the ligated RNA sample:
   a. Add 16 µL of the RT master mix to each 20-µL ligation reaction.
   b. Gently vortex the reaction to mix thoroughly, then centrifuge the reaction briefly to collect the liquid in the bottom of the tube.
   c. Incubate in a thermal cycler with a heated lid at 70°C for 10 minutes, then snap-cool on ice.
3. Perform the reverse transcription reaction:
   a. Add 4 µL of 10X SuperScript® III Enzyme Mix to each ligated RNA sample.
   b. Gently vortex to mix thoroughly, then centrifuge briefly.
   c. Incubate in a thermal cycler with a heated lid at 42°C for 30 minutes.

STOPPING POINT The cDNA can be stored at –30°C to –10°C for 2 weeks, stored at –86°C to –68°C for long-term storage, or used immediately.

Purify the cDNA

Required materials from the Magnetic Bead Cleanup Module

- Wash Solution Concentrate
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate
- Nuclease-Free Water

Other materials and equipment

- 100% ethanol or 200 proof (absolute) ethanol, ACS-grade or higher quality
- Magnetic stand for 96-well plates (Life Technologies, Cat. no. AM10027 or AM10050)
- 37°C heat block or water bath
- (*Optional*) MicroAmp® Clear Adhesive Film (Life Technologies, Cat. no. 4306311)

Before you begin

- If you have not done so already, add 44 mL of 100% ethanol to the Wash Solution Concentrate and mix well. Mark the label on the bottle to indicate that you added ethanol. Store the solution at room temperature (15°C to 30°C)
- If you see a white precipitate in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Nuclease-Free Water at 37°C for ≥5 minutes.

Note: To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp® Clear Adhesive Film (Life Technologies, Cat. no. 4306311). You can also skip a row between sample rows.

IMPORTANT! Accurate pipetting of bead cleanup reagents is critical for best results.

1. Prepare beads for each sample:
    a. Gently vortex the Nucleic Acid Binding Beads tube to completely resuspend the magnetic beads.
    b. Add 5 µL beads to wells on the Processing Plate.
    c. Add 120 µL Binding Solution Concentrate to each well, then mix the Binding Solution Concentrate and beads by pipetting up and down 10 times.
2. Bind the cDNA to the beads:
    a. Add 60 µL of Nuclease-Free Water to each of the 40-µL RT reaction.
    b. Transfer each 100-µL RT reaction to a bead-containing well on the Processing Plate.
    c. Set a P200 pipettor at 125 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the new 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times.

d. Without changing tips, add 125 µL of 100% ethanol to each well.

IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 2c–2d for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

e. Set a single or multi-channel pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

Note: The color of the mixture should be homogeneous after mixing.

f. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

3. Remove the supernatant from the beads:
   a. Place the Processing Plate on the magnetic stand for 5–6 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
   b. Leave the Processing Plate on the magnetic stand, then aspirate and discard the supernatant from the plate.

IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 microliters of supernatant behind.

4. Wash the beads with Wash Solution Concentrate with ethanol:
   a. Leave the Processing Plate on the magnetic stand.
   b. Add 150 µL of Wash Solution Concentrate with ethanol to each sample.
   c. Incubate the samples at room temperature for 30 seconds.
5. Remove the supernatant from the beads:
   a. Aspirate and discard the supernatant from the plate.
   b. Use a P10 or P20 pipettor to remove residual ethanol.

IMPORTANT! Do not disturb the separated magnetic beads. Remove all of the Wash Solution Concentrate from each well.

   c. Air-dry the beads at room temperature to remove all traces of ethanol by leaving the Processing Plate on the magnetic stand for 1–2 minutes.

IMPORTANT! Do not overdry the beads (overdried beads appear cracked). Overdrying significantly decreases elution efficiency.

6. Elute the cDNA from the beads:
   a. Remove the Processing Plate from the magnetic stand.
   b. Add 12 µL of pre-warmed (37°C) Nuclease-Free Water to each sample, then mix the Nuclease-Free Water and beads by pipetting up and down 10 times.
   c. Incubate at room temperature for 1 minute.
   d. Place the Processing Plate on the magnetic stand for 1 minute to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

e. For each sample, collect the eluant.

Amplify the cDNA

To prepare non-barcoded libraries, use the following components from the Ion Total RNA-Seq Kit v2:

- Ion 5′ PCR Primer v2
- Ion 3′ PCR Primer v2
- Platinum® PCR SuperMix High Fidelity

To prepare barcoded libraries, plan the barcodes that you want to use, then select the PCR primers from Ion Xpress™ RNA-Seq Barcode 01–16 Kit:

- Ion Xpress™ RNA-Seq Barcode BC 01–BC 16
- Ion Xpress™ RNA 3′ Barcode Primer

1. For each cDNA sample, prepare the PCR mix, according to the non-barcoded or barcoded library tables.

IMPORTANT! Use the appropriate primers.

Non-barcoded Library	
Component	**Volume for One Reaction†**
Platinum® PCR SuperMix High Fidelity‡	45 µL
Ion 5′ PCR Primer v2	1 µL
Ion 3′ PCR Primer v2	1 µL
Total volume	**47 µL**

† Include 5–10% excess volume to compensate for pipetting error when preparing master mix.

‡ Platinum® PCR SuperMix High Fidelity contains a proofreading enzyme for high-fidelity amplification.

a. Transfer 6 µL of cDNA sample to a new PCR tube.

b. Transfer 47 µL of the PCR mix to each 6 µL of cDNA sample.

c. Proceed to step 2.

Barcoded Library	
Component	**Volume for One Reaction†**
Platinum® PCR SuperMix High Fidelity‡	45 µL
Ion Xpress™ RNA 3′ Barcode Primer	1 µL
Total volume	**46 µL**

† Include 5–10% excess volume to compensate for pipetting error when preparing master mix.

‡ Platinum® PCR SuperMix High Fidelity contains a proofreading enzyme for high-fidelity amplification.

a. Transfer 6 µL of cDNA sample to a new PCR tube.

b. Transfer 46 µL of the PCR mix to each 6 µL of cDNA sample.

c. Add 1 µL of the selected Ion Xpress™ RNA-Seq Barcode BC primer (choose from BC01–BC16) to each PCR tube.

d. Proceed to step 2.

2. Flick the tube or slowly pipet the solution up and down 5 times to mix well, then centrifuge briefly to collect the liquid in the bottom of the tube.

3. Run the PCRs in a thermal cycler:

Stage	Temp	Time
Hold	94°C	2 min
Cycle (2 cycles)	94°C	30 sec
	50°C	30 sec
	68°C	30 sec
Cycle • 16 cycles for 1–5 ng poly(A) RNA or 10–100 ng rRNA-depleted RNA • 14 cycles for >5 ng poly(A) RNA or >100 ng rRNA-depleted RNA	94°C	30 sec
	62°C	30 sec
	68°C	30 sec
Hold	68°C	5 min

Purify the amplified cDNA

Required materials from the Magnetic Bead Cleanup Module

- Wash Solution Concentrate
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate
- Nuclease-Free Water

Other materials and equipment

- 100% ethanol or 200 proof (absolute) ethanol, ACS-grade or higher quality
- Magnetic stand for 96-well plates (Life Technologies, Cat. no. AM10027 or AM10050)
- 37°C heat block or water bath
- (*Optional*) MicroAmp® Clear Adhesive Film (Life Technologies, Cat. no. 4306311)

Before you begin

- If you have not done so already, add 44 mL of 100% ethanol to the Wash Solution Concentrate and mix well. Mark the label on the bottle to indicate that you added ethanol. Store the solution at room temperature (15°C to 30°C).
- If you see a white precipitate in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Nuclease-Free Water at 37°C for ≥5 minutes.

Note: To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp® Clear Adhesive Film (Life Technologies, Cat. no. 4306311). You can also skip a row between sample rows.

IMPORTANT! Accurate pipetting of bead cleanup reagents is critical for best results.

1. Prepare beads for each sample:
    a. Gently vortex the Nucleic Acid Binding Beads tube to completely resuspend the magnetic beads.
    b. Add 5 µL beads to wells on the Processing Plate.
    c. Add 180 µL Binding Solution Concentrate to each well, then mix the Concentrate and beads by pipetting up and down 10 times.
2. Bind the amplified cDNA to the beads:
    a. Transfer 53 µL of each amplified cDNA sample to a bead-containing well on the Processing Plate.
    b. Set a P200 pipettor at 130 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the new 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times.
    c. Without changing tips, add 130 µL of 100% ethanol to each well.

    IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 2b–2c for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

    d. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

    Note: The color of the mixture should be homogeneous after mixing.

    e. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.
3. Remove the supernatant from the beads:
    a. Place the Processing Plate on a magnetic stand for 5–6 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
    b. Leave the Processing Plate on the magnetic stand, then aspirate and discard the supernatant from the plate.

    IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 microliters of supernatant behind.

4. Wash the beads with Wash Solution Concentrate with ethanol:
    a. Leave the Processing Plate on the magnetic stand.
    b. Add 150 µL of Wash Solution Concentrate with ethanol to each sample.
    c. Incubate the samples at room temperature for 30 seconds.
5. Remove the supernatant from the beads:
    a. Aspirate and discard the supernatant from the plate.

b. Use a P10 or P20 pipettor to remove residual ethanol.

IMPORTANT! Do not disturb the separated magnetic beads. Remove all of the Wash Solution Concentrate from each well.

c. Air-dry the beads at room temperature to remove all traces of ethanol by leaving the Processing Plate on the magnetic stand for 1–2 minutes.

IMPORTANT! Do not overdry the beads (overdried beads appear cracked). Overdrying significantly decreases elution efficiency.

6. Elute the cDNA from the beads:
   a. Remove the Processing Plate from the magnetic stand.
   b. Add 15 µL of pre-warmed (37°C) Nuclease-Free Water to each sample, then mix the Nuclease-Free Water and beads by pipetting up and down 10 times.
   c. Incubate at room temperature for 1 minute.
   d. Place the Processing Plate on a magnetic stand for 1 minute to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
   e. For each sample, collect the eluant.

Assess the yield and size distribution of the amplified DNA

Use a NanoDrop® Spectrophotometer or the dsDNA HS Assay Kit with the Qubit® Fluorometer. Also use the Agilent® 2100 Bioanalyzer® instrument with the Agilent® DNA 1000 Kit or Agilent® High Sensitivity DNA Kit.

1. Measure the concentration of the purified DNA with a NanoDrop® Spectrophotometer or the dsDNA HS Assay Kit with the Qubit® Fluorometer.
2. Analyze 1 µL of the library using the appropriate chip on the Agilent® 2100 Bioanalyzer® instrument. If the library concentration is:
   - 1–50 ng/µL: Use the Agilent® DNA 1000 Kit.
   - 5–1000 pg/µL: Use the Agilent® High Sensitivity DNA Kit.
3. Using the 2100 expert software, perform a smear analysis to:
   a. Quantify the percentage of DNA that is ≤160 bp: Use size range 50–160 bp.
   b. Determine the molar concentration (nM) of the cDNA libraries: Use size range 50–1000 bp.

   Note: For instructions on how to perform the smear analysis, see "Perform a smear analysis" on page 68, and refer to the *Agilent® 2100 Bioanalyzer® Expert User's Guide* (Pub. no. G2946-90000).
4. Use molar concentration of the cDNA libraries from 3b of "Pool barcoded whole transcriptome libraries" on page 33 and "Determine the library dilution required for template preparation" on page 34.

Next steps:

If the percent of DNA in 50–160 bp the range is...	Then...
<50%	Proceed to "Determine the library dilution required for template preparation" on page 34 or "Pool barcoded whole transcriptome libraries" on page 33.
50–60%	Perform another round of purification on the amplified DNA using components from the Magnetic Bead Cleanup Module: 1. Bring the sample volume to 53 µL with Nuclease-Free Water. 2. Follow the steps in "Purify the cDNA" on page 27. *or* Proceed to "Determine the library dilution required for template preparation" on page 34 or "Pool barcoded whole transcriptome libraries", but expect to see a slightly higher percentage of filtered reads in your sequencing data when compared to libraries with less than 50% of DNA in the range.
>60%	We recommend that you perform another round of purification on the amplified DNA using components from the Magnetic Bead Cleanup Module: 1. Bring the sample volume to 53 µL with Nuclease-Free Water. 2. Follow the steps in "Purify the cDNA" on page 27.

Pool barcoded whole transcriptome libraries

Note: If you are not pooling libraries, skip this section and proceed to "Determine the library dilution required for template preparation" on page 34.

1. Determine the molar concentration (nM) of each of the barcoded cDNA libraries with the Agilent® DNA 1000 Kit or the Agilent® High Sensitivity DNA Kit.

   Note: 50–1000 bp size range is typically used to determine library concentration. If necessary, adjust the range to include all the library peaks.

2. Dilute each barcoded cDNA library to the same molar concentration (nM). For example, if you have 3 different barcoded libraries that are 45, 55, 65 nM, choose a concentration that is equal to or lower than the lowest concentration of the three libraries, such as 30 nM. Dilute all or part of the library to 30 nM.

3. Mix an equal volume of each diluted library to prepare a pool of the barcoded libraries.

4. The final molar concentration of the pooled library is the same for each diluted library. For example, if you dilute each library to 30 nM, the concentration of the pooled library is 30 nM.

   Use the final molar concentration to determine the Template Dilution Factor. You can also determine the molar concentration of the pooled libraries with the Agilent® DNA 1000 Kit or the Agilent® High Sensitivity DNA Kit (see "Assess the yield and size distribution of the amplified DNA" on page 32 and "Using 2100 expert software to assess whole transcriptome libraries" on page 68).

Determine the library dilution required for template preparation

With less than 50% of the amplified DNA in the correct range, you can proceed to the template preparation procedure (see "Proceed to template preparation") to prepare templated beads for sequencing on the Ion PGM™ System or the Ion Proton™ System.

For template preparation using an appropriate Ion template preparation kit, determine the library dilution that gives 210×10^6 molecules of template per 20 µL; 210×10^6 is the recommended library input for Whole Transcriptome libraries. Use a conversion factor of 8.3 nM = 5×10^9 molecules/µL, and the following formula:

Template Dilution Factor =
(Library concentration in nM) × [(5 × 10^9 molecules/µL)/(8.3 nM)] × [(volume per template preparation reaction inµL)/(210 × 10^6 molecules)]

For the volume per template preparation reaction, refer to the specific user guide for the appropriate Ion template preparation kit.

If your library concentration is >20 nM, serially dilute 1:10 or 1:100 until your library concentration is ≤20 nM. (This step helps prevent a Template Dilution Factor of several thousands).

Example: The whole transcriptome RNA library concentration is 400 nM. Volume per template preparation reaction is 20 µL.

Dilute 1 µL of library in 99 µL of Nuclease-Free Water (1:100 dilution) to yield a final library concentration of 4 nM. Use this final library concentration to calculate the Template Dilution Factor:

Template Dilution Factor =
(4 nM) × [(5 × 10^9 molecules/µL)/(8.3 nM)] × [20 µL/(210 × 10^6 molecules)] = 229

Thus, 1 µL of the 4-nM library dilution mixed with 228 µL of Nuclease-Free Water (1:229 dilution) yields approximately 210×10^6 molecules per 20 µL.

Proceed to template preparation

The library is ready for the template preparation procedure. In this procedure, each library template is clonally amplified on Ion Sphere™ Particles for sequencing on the Ion PGM™ System or Ion PI™ Ion Sphere™ Particles for sequencing on the Ion Proton™ System. For instructions, refer to the specific user guide for an appropriate Ion template preparation kit.

Template preparation documentation is available on the Ion Community at **http://ioncommunity.iontorrent.com/**. Follow the links under **Protocols** > **Prepare Template** > **Prepare Template User Guides and Quick Reference**. You may also refer to the *Preparation and Sequencing of RNA Libraries with the Ion Personal Genome Machine® (PGM™) System User Bulletin* (Pub. no. 4478119).

Size distributions and yields

Typical size distributions

The highest quality libraries have less than 50% amplified DNA between 25–160 bp:

Figure 3 Molar concentration and size distribution of amplified library prepared from HeLa poly(A) RNA

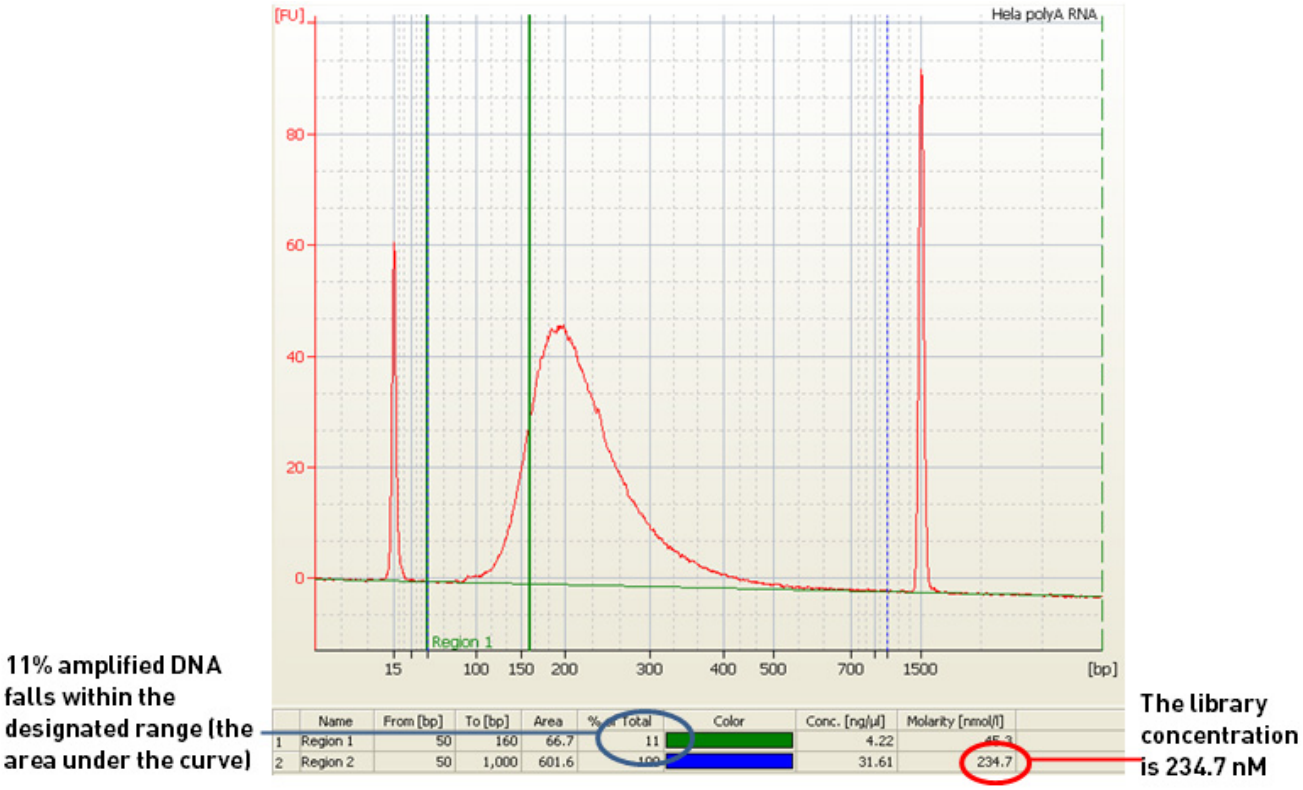


Figure 4 Molar concentration and size distribution of amplified library prepared from HeLa rRNA-depleted total RNA

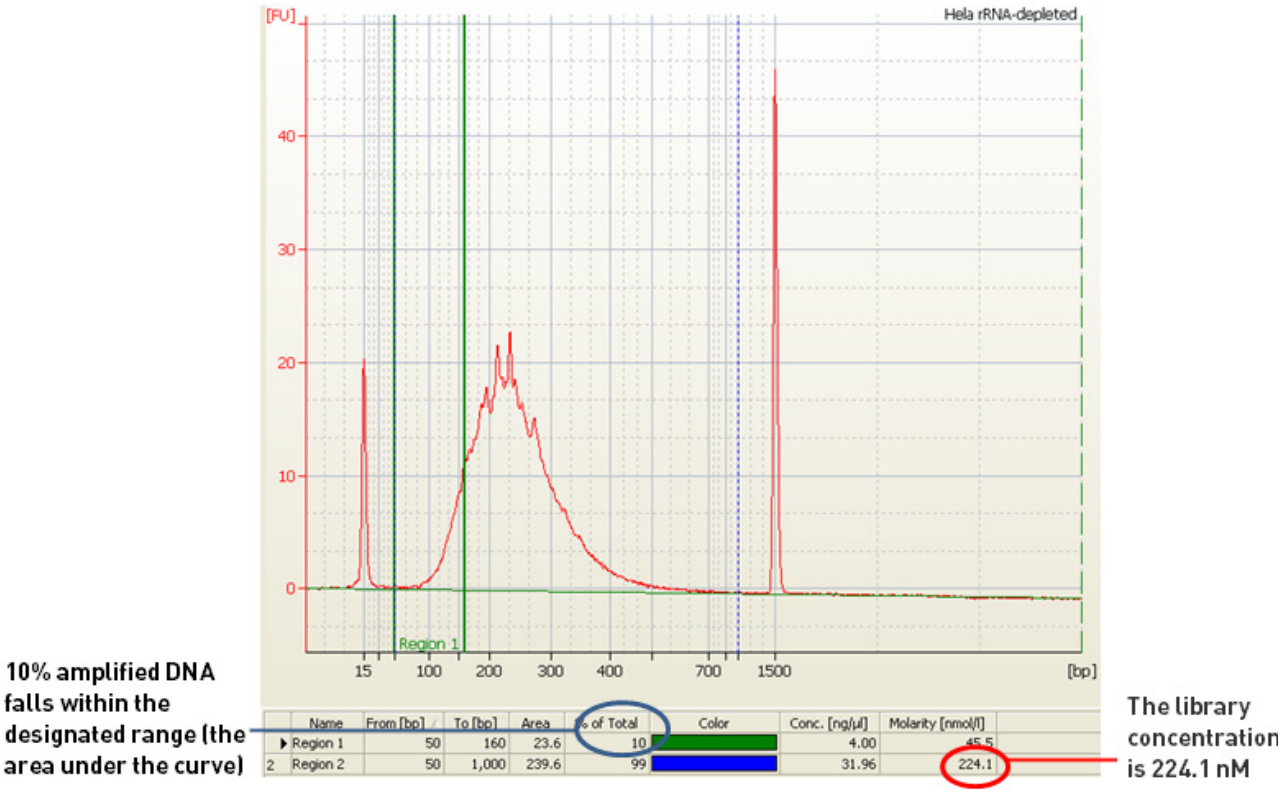
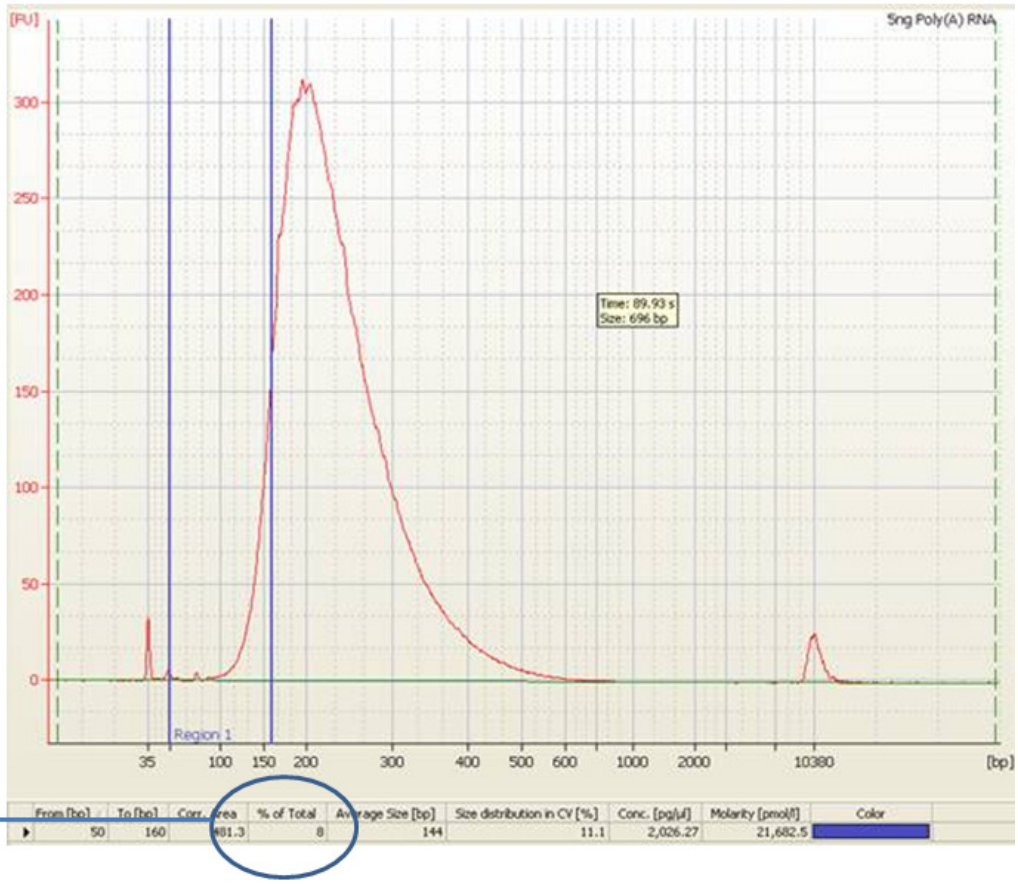
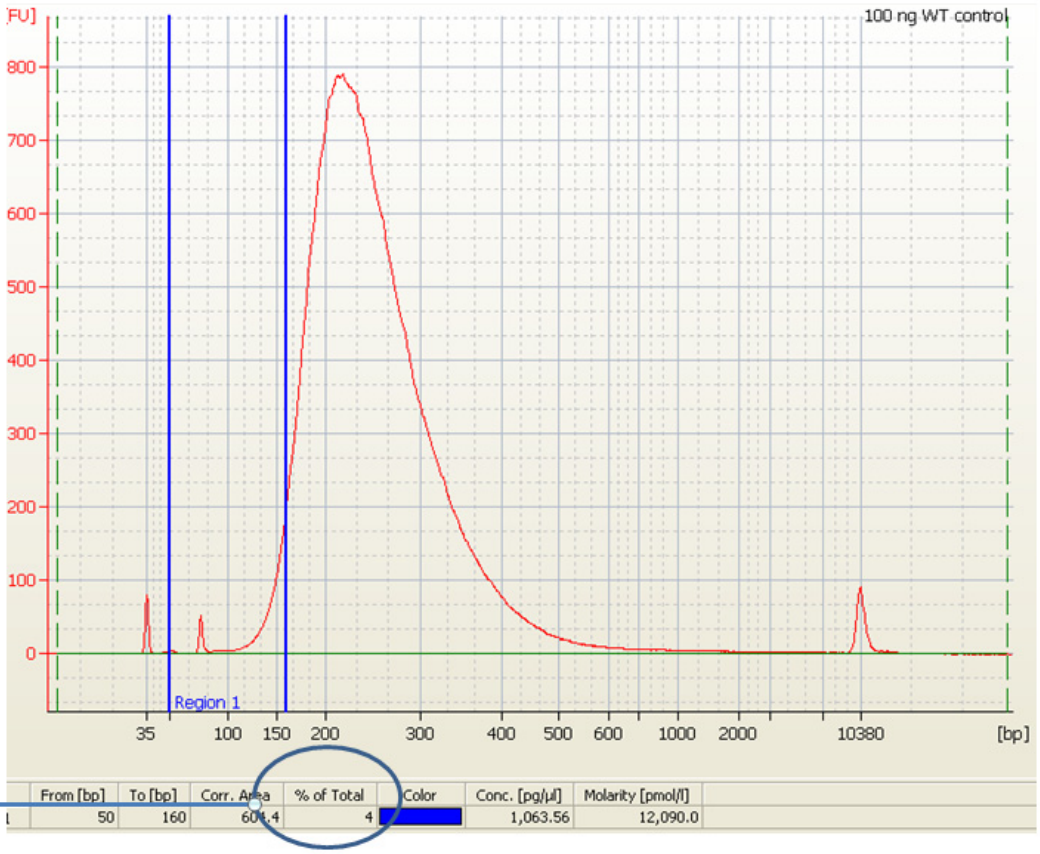

[FU]
Hela rRNA-depleted
40
30
20
10
0
Region 1
15
100
200
300
400
700
1500
[bp]
Name | From [bp] | To [bp] | Area | % of Total | Color | Conc. [ng/µl] | Molarity [nmol/l]
Region 1 | 50 | 160 | 23.6 | 10 | | 4.00 | 45.5
2 Region 2 | 50 | 1,000 | 239.6 | 99 | | 31.96 | 224.1
10% amplified DNA falls within the designated range (the area under the curve)
The library concentration is 224.1 nM

Figure 5 Size distribution of amplified library prepared using 5 ng of poly(A) RNA and isolated using the Dynabeads® mRNA DIRECT™ Micro Kit. Refer to "Poly(A) selection from 100 ng–1µg input Total RNA samples" protocol in the *Dynabeads® mRNA DIRECT™ Micro Kit User Guide*.



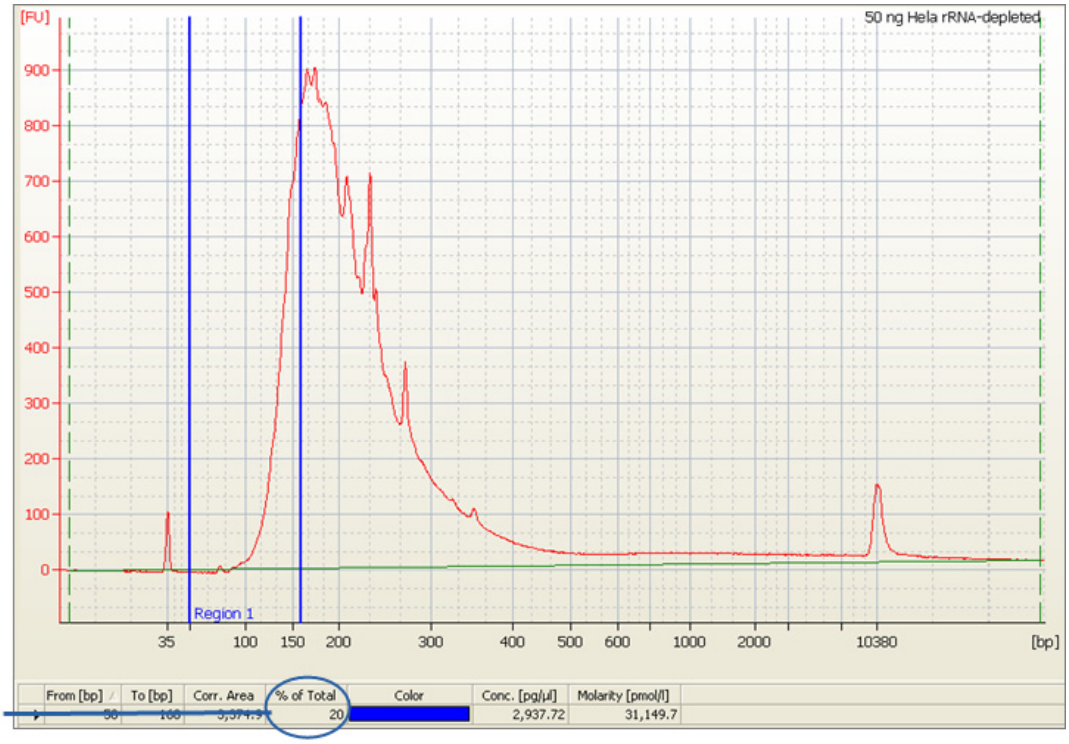
8% amplified DNA falls within the designated range (the area under the curve)

Figure 6 Size distribution of amplified library prepared using poly(A) RNA from 100 ng WT Control RNA



4% amplified DNA falls within the designated range (the area under the curve)

Figure 7 Size distribution of amplified library prepared from 50 ng of rRNA-depleted total RNA using the Agilent® High Sensitivity DNA Kit



20% of amplified DNA falls within the designated range (the area under the curve)

Expected yields

The recovery of your experimental RNA will depend on its source and quality. The following results are typically seen with Human Brain Reference and HeLa RNAs.

Note: Typical amplified DNA yields for HeLa poly(A) RNA and HeLa rRNA-depleted total RNA are greater than 200 ng in a 15-µL final volume.

Workflow	Input Amount	Typical Recovery Amount
Fragment the whole transcriptome RNA (page 17)	500 ng of poly(A) RNA, total RNA, or rRNA-depleted total RNA	300–400 ng of RNA
Construct the whole transcriptome library (page 24)	<1–100 ng of fragmented RNA	>5 ng of cDNA

Troubleshooting

Observation	Possible Cause	Solution
The Agilent® software does not calculate one concentration and peak size	The software detects multiple peaks in the amplified cDNA profile	Refer to "Analyze multiple peaks as one peak" on page 69.
Low yield and poor size distribution in the amplified library	You recovered <20% of the input RNA after you fragmented and cleaned up the RNA	Decrease the RNase III digestion from 10 minutes to 5 minutes (step 3 on page 19).
Low yield in the amplified library and very few differences in the Agilent® 2100 Bioanalyzer® instrument traces before and after you fragment the RNA	RNA fragmentation failed	Purify the RNA sample again to remove the extra salts that may affect the RNase III activity. If RNA fragmentation still fails, increase the RNase III digestion from 10 minutes to 20 minutes (step 3 on page 19).
Low yield and no PCR products	An enzymatic reaction or purification performed after RNase III treatment failed	Repeat the ligation with more fragmented RNA, and run a parallel ligation reaction with fragmented Control RNA.

Using a positive control to troubleshoot

A general troubleshooting strategy is to perform the Ion Total RNA-Seq Kit v2 procedure using the WT Control RNA (HeLa total RNA) provided with the kit.

- Use 500 ng of WT Control RNA for the fragmentation procedure starting on page 17.
- Use 100 ng of fragmented WT Control RNA in the amplified library construction procedure starting on page 24.

The expected yields for the WT Control RNA should be the same as the RNA and cDNA yields listed on page 39.

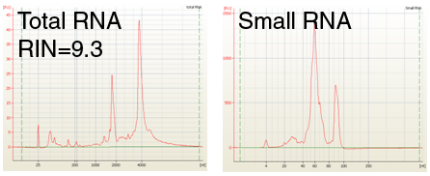
3 Prepare Small RNA Libraries

- Workflow . . . 42
- Prepare the starting material . . . 43
  - Guidelines for obtaining small RNA . . . 43
  - Assess the amount and quality of small RNA in your total RNA samples . . . 43
  - Enrich the samples for small RNA . . . 44
  - Assess the quality and quantity of the small RNA-enriched sample . . . 48
  - Determine the input amount . . . 48
- Construct the small RNA library . . . 49
  - Hybridize and ligate the RNA . . . 49
  - Perform reverse transcription . . . 51
  - Purify and size-select the cDNA . . . 52
  - Amplify the cDNA . . . 54
  - Purify and size-select the amplified DNA . . . 56
  - Assess the yield and size distribution of the amplified DNA . . . 59
- Pool barcoded small RNA libraries . . . 60
- Determine the library dilution required for template preparation . . . 60
- Proceed to template preparation . . . 61
- Typical size distribution . . . 61
  - Plotted size distributions . . . 61
  - Size distributions compared . . . 64
- Troubleshooting . . . 65
  - Using a positive control to troubleshoot . . . 65

Workflow

Prepare the starting material

Assess the amount and quality of small RNA in your total RNA samples (page 43)



▼

Enrich the samples for small RNA (page 44)

▼

Assess the quality and quantity of the small RNA-enriched sample (page 48)

▼

Determine the input amount (page 48)

▼

Construct the small RNA library

Hybridize and ligate the RNA (page 49)

▼

Perform reverse transcription (page 51)

▼

Purify and size-select the cDNA (page 52)

▼

Amplify the cDNA (page 54)

▼

Purify and size-select the amplified DNA (page 56)

▼

Assess the yield and size distribution of the amplified DNA (page 59)

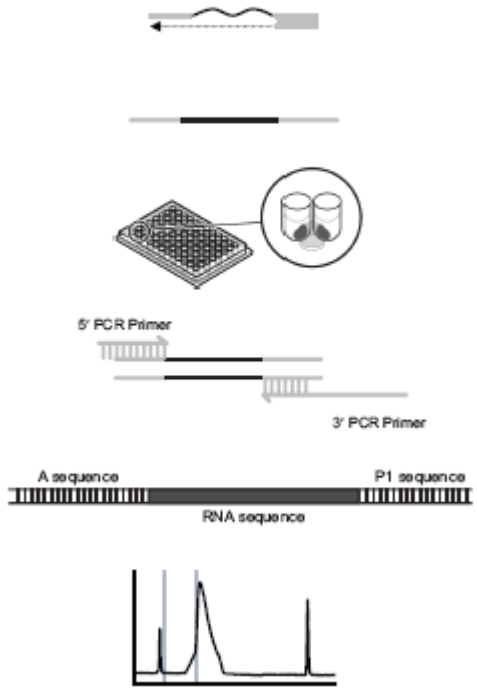
▼

Pool barcoded small RNA libraries (page 60)

▼

Determine the library dilution required for template preparation (page 60)

▼



Prepare templated Ion Sphere™ particles for sequencing on the Ion PGM™ System

Refer to the end user documentation for the Ion OneTouch™ System (Pub. no. 4478372)

Prepare the starting material

Preparing the starting material involves the following procedures:

Assess the amount and quality of small RNA in your total RNA samples (page 43)

⬇

Enrich the samples for small RNA (page 44)

⬇

Assess the quality and quantity of the small RNA-enriched sample (page 48)

Guidelines for obtaining small RNA

For this protocol, the total RNA must contain the small RNA fraction (microRNA or miRNA, 10–40 nt). For optimal results, use RNA that has been size-selected for small RNA.

We recommend the following products:

- RNA source: Best results are obtained using high quality total RNA with an RNA integrity number (RIN) >6 as your starting material.
- RNA isolation kits: Use the mirVana™ miRNA Isolation Kit or the mirVana™ PARIS™ Kit to isolate total RNA that includes the small RNA fraction or small RNA from tissue or cells. Use the PureLink® miRNA Isolation Kit to isolate small RNA from tissues or cells.

Assess the amount and quality of small RNA in your total RNA samples

Before you prepare the library, determine the quality of the total RNA sample. Use the NanoDrop® Spectrophotometer and the Agilent® 2100 Bioanalyzer® instrument with the Agilent® RNA 6000 Nano Kit and the Agilent® Small RNA Kit.

1. Quantitate the amount of RNA in the sample using the NanoDrop® Spectrophotometer.

   Note: If you used the mirVana™ miRNA Isolation Kit, the mirVana™ PARIS™ Kit, or the PureLink® miRNA Isolation Kit to isolate small RNA from samples, you can skip to "Assess the quality and quantity of the small RNA-enriched sample" on page 48.

2. Determine the quality of the small RNA in your sample:

   Note: For instructions on using the software, refer to the *Agilent® 2100 Bioanalyzer® Expert User's Guide* (Pub. no. G2946-9000).

   a. Dilute the RNA to ~50–100 ng/µL.

   b. Run 1 µL of diluted RNA on the Agilent® 2100 Bioanalyzer® instrument with the Agilent® RNA 6000 Nano chip to determine the concentration of total RNA. Follow the manufacturer's instructions for performing the assay.

   c. Using the 2100 expert software, determine the mass of total RNA in the sample, and save the mass of total RNA for step 3c to calculate the miRNA content.

d. Using the 2100 expert software, review the RNA Integrity Number (RIN). The highest quality library mapping statistics are obtained from input RNA with higher RIN values.

3. Determine the percentage of small RNA in your sample:
   a. Run 1 µL of diluted RNA on the Agilent ® 2100 Bioanalyzer® instrument with the Small RNA Kit chip. Follow the manufacturer's instructions for performing the assay.
   b. Using the 2100 expert software, determine the mass of total RNA (miRNA; 10–40 nt) from the Small RNA Kit chip.
   c. Calculate the miRNA content in your RNA sample using the formula:
      % miRNA = (mass of miRNA ÷ mass of total RNA) × 100

4. Determine whether small RNA enrichment is needed and the type of enrichment to perform:

How much miRNA (10–40 nt) is in your RNA sample?	Recommendations for small RNA enrichment and next steps
≥0.5% miRNA	You can use the total RNA in the ligation reaction, and small RNA enrichment is not needed. However, for optimal results, we recommend enrichment of all total RNA samples. You can expect to see 5–15% more of rRNA and tRNA mapping in your sequencing data from total RNA, compared to sequencing data of libraries starting from enriched small RNA. Proceed to "Enrich the samples for small RNA" or skip to "Determine the input amount" on page 48.
<0.5% miRNA	Small RNA enrichment is strongly recommended. We recommend using the Magnetic Bead Cleanup Module for small RNA enrichment. Proceed to "Enrich the samples for small RNA".

Enrich the samples for small RNA

Note: We recommend enriching all total RNA samples for small RNA for optimal results. However, if the tissue or cell lines contain sufficient small RNA to allow efficient library preparation, skip to "Assess the quality and quantity of the small RNA-enriched sample" on page 48.

Based on their source and the RNA isolation method, RNA samples vary widely in small RNA content. In some tissues, the proportion of small RNA is high enough to allow efficient library preparation from total RNA. For example, the control RNA provided in this kit is total RNA isolated from placenta. Many tissues and most cell lines, however, contain a much smaller fraction of small RNA.

If the tissues or cell lines that you are using contain a small fraction of small RNA, we recommend enrichment of these RNA samples for small RNA. Use an Agilent® Small RNA chip to measure the concentration of miRNA (10-40 nts) in your total RNA or enriched small RNA sample.

This protocol uses magnetic beads to enrich for small RNA. Use the Magnetic Bead Cleanup Module twice with the same sample to size-select the desired small RNA products. During the first bead binding, magnetic beads capture larger RNA species such as mRNA and rRNA. During the second binding and with increased ethanol concentration, desired small RNA products (miRNA and other small RNA) in the supernatant re-bind to the magnetic beads. After washing the beads, the desired small RNA products are eluted with pre-warmed (80°C) Nuclease-Free Water.

Required materials from the Magnetic Bead Cleanup Module

- Wash Solution Concentrate
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate
- Nuclease-Free Water

Note: The 1.5-mL Non-Stick RNAse-free Microfuge Tubes (Cat. no. AM12450), may be used in place of the Processing Plate.

Other materials and equipment

- 100% ethanol or 200 proof (absolute) ethanol, ACS-grade or higher quality
- Magnetic rack or stand
- 80°C heat block or water bath
- (*Optional*) MicroAmp® Clear Adhesive Film

Before you begin

- Add 44 mL of 100% ethanol to the bottle of Wash Solution Concentrate and mix well. Mark the label on the bottle to indicate that you added ethanol. Store the solution at room temperature (15°C to 30°C).
- If you see a white precipitate in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Nuclease-Free Water at 80°C for ≥5 minutes.

Note: To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp® Clear Adhesive Film (Life Technologies, Cat. no. 4306311). You can also skip a row between sample rows.

IMPORTANT! Accurate pipetting of bead cleanup reagents is critical to successful size-selection. For optimal size-selection, perform the following bead cleanup steps exactly.

---

1. Prepare beads for each sample:
    a. Gently vortex the Nucleic Acid Binding Beads tube to completely resuspend the magnetic beads.
    b. Add 7 µL beads to the wells of the Processing Plate.
    c. Add 120 µL Binding Solution Concentrate to each well, then mix the Concentrate and beads by pipetting up and down 10 times.
2. Bind larger RNA to the beads:

a. Resuspend 0.5–20 µg of total RNA in 75 µL Nuclease-Free Water.

b. Transfer 75 µL of each RNA sample to a well with beads of the Processing Plate.

c. Set a P200 pipettor at 105 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the new 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times.

d. Without changing tips, add 105 µL of 100% ethanol to each well.

IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 2c–2d for the remaining wells only if the tip touches the wall Accurate pipetting of 100% ethanol is critical for size-selection. Follow the instructions exactly for best results.

---

e. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

Note: The color of the mixture should be homogeneous after mixing.

f. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

3. Remove the supernatant from the beads:

a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

b. Leave the Processing Plate on the magnetic stand, then transfer the supernatant to a new well on the plate or to a well on a new plate.

IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 microliters of supernatant behind.

---

4. Bind desired small RNA products to the beads:

a. Remove the Processing Plate from the magnetic stand.

b. Add 30 µL of Nuclease-Free Water to the supernatant in the new sample well.

c. Set a P1000 pipettor at 570 µL. Attach a new 1000-µL tip to the pipettor, then pre-wet the new 1000-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times.

d. Without changing tips, add 570 µL of 100% ethanol to each well.

IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 4c–4d for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for size-selection. Follow the instructions exactly for best results.

---

e. Gently vortex the Nucleic Acid Binding Beads tube to completely resuspend the magnetic beads.

f. Add 7 µL beads to the wells of the Processing Plate.

g. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

IMPORTANT! Due to the large volume in each well, use a P200 pipettor for mixing to avoid cross-well contamination.

Note: The color of the mixture should be homogeneous after mixing.

h. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

5. Remove the supernatant from the beads:

a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

b. Leave the Processing Plate on the magnetic stand, then carefully aspirate and discard the supernatant from the plate.

IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 microliters of supernatant behind.

---

6. Wash the beads with the Wash Solution Concentrate with ethanol:

a. Leave the Processing Plate on the magnetic stand, then add 150 µL of Wash Solution Concentrate with ethanol to each sample.

b. Incubate the samples for 30 seconds.

c. Leave the Processing Plate on the magnetic stand, then aspirate and discard the supernatant from the plate.

IMPORTANT! Do not disturb the separated magnetic beads. Remove all of the Wash Solution Concentrate from each well.

---

d. Use a P10 or P20 pipettor to remove residual ethanol.

e. Air-dry the beads at room temperature for 1–2 minutes to remove all traces of ethanol.

IMPORTANT! Do not overdry the beads (overdried beads appear cracked); overdrying significantly decreases elution efficiency.

---

7. Elute the small RNA from the beads:

a. Remove the Processing Plate from the magnetic stand.

b. Add 30 µL of pre-warmed (80°C) Nuclease-Free Water to each sample.

c. Mix thoroughly by pipetting up and down 10 times.

d. Incubate the samples at room temperature) for 1 minute.

e. Place the Processing Plate on the magnetic stand for 1 minute to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

f. For each sample, collect 30 µL of the eluant.

STOPPING POINT Store the small RNA at –86°C to –68°C. After storage, proceed to the next section "Assess the quality and quantity of the small RNA-enriched sample".

Assess the quality and quantity of the small RNA-enriched sample

Assess the quality and quantity of samples that are enriched for small RNA. Use the Agilent® 2100 Bioanalyzer® instrument with the Agilent® Small RNA Kit.

1. Run 1 µL of purified and *enriched small RNA* sample on the Agilent® 2100 Bioanalyzer® instrument with the Small RNA Kit chip. Follow the manufacturer's instructions for performing the assay.
2. Compare the bioanalyzer traces to those of the sample before enrichment (see step 2 in "Assess the amount and quality of small RNA in your total RNA samples" on page 43), and determine whether the RNA is degraded. For enriched small RNA samples, peaks should be from 10–200 nt.

Determine the input amount

Using the results from the Agilent® 2100 Bioanalyzer® instrument and the Small RNA Kit, determine the amount of total RNA to use according to the type of RNA you ran and the amount of miRNA in 3 µL. If necessary, concentrate the small RNA with a SpeedVac® centrifugal concentrator.

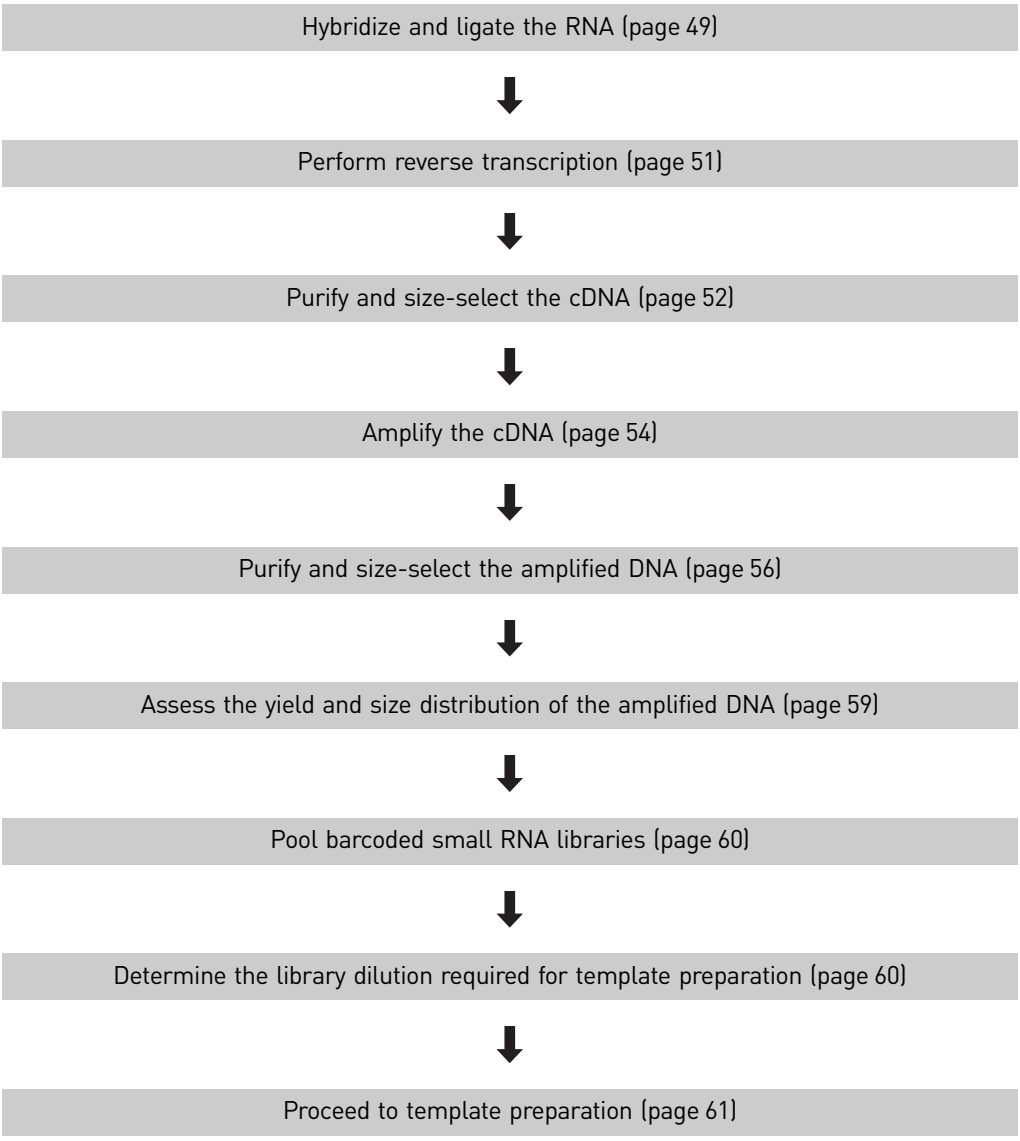
Input Sample Type	Amount of miRNA (10–40 nt) in 3 µL	Total RNA Input†
Total RNA	5–100 ng	≤1 µg
Enriched small RNA	1–100 ng	≤1 µg

† The yield drops if you use more than 1 µg of RNA for ligation.

Note: When starting from total RNA with low RIN, the miRNA quantity could be over-estimated on the Agilent® Small RNA chip, so more input into ligation is recommended. Ideally, use >10 ng of miRNA.

Construct the small RNA library

Constructing the amplified small RNA library involves the following procedures:


Hybridize and ligate the RNA (page 49)
Perform reverse transcription (page 51)
Purify and size-select the cDNA (page 52)
Amplify the cDNA (page 54)
Purify and size-select the amplified DNA (page 56)
Assess the yield and size distribution of the amplified DNA (page 59)
Pool barcoded small RNA libraries (page 60)
Determine the library dilution required for template preparation (page 60)
Proceed to template preparation (page 61)

Hybridize and ligate the RNA

Required materials from the Ion Total RNA-Seq Kit v2

- Ion Adaptor Mix v2
- Hybridization Solution
- 2X Ligation Buffer
- Ligation Enzyme Mix

1. On ice, prepare the hybridization master mix:

Component	Volume for One Reaction†
Hybridization Solution	3 µL
Ion Adaptor Mix v2	2 µL
Total volume	**5 µL**

† Include 5–10% excess volume to compensate for pipetting error when preparing the master mix.

2. Add 5 µL of hybridization master mix to 3 µL of small RNA sample (1–100 ng of miRNA in ≤1 µg of enriched small RNA).
3. Flick the tube or pipet up and down 5 times to mix, then centrifuge briefly to collect the liquid in the bottom of the tube.
4. Run the hybridization reaction in a thermal cycler:

Temperature	Time
65°C	10 min
16°C	5 min

5. On ice, prepare the ligation master mix:
   a. Combine in a 0.5-mL or 1.5-mL Non-Stick RNase-Free Microfuge Tube:

Component	Volume for One Reaction†
2X Ligation Buffer	10 µL
Ligation Enzyme Mix	2 µL
Total volume	**12 µL**

† Include 5–10% excess volume to compensate for pipetting error when preparing the master mix.

IMPORTANT! If the 2X Ligation Buffer contains a white precipitate, warm the tube at 37°C for 2–5 minutes or until the precipitate is dissolved. 2X Ligation Buffer is very viscous; pipet slowly to dispense it accurately.

   b. Gently vortex to mix, then centrifuge briefly to collect the liquid in the bottom of the tube.
6. Add 12 µL of ligation master mix to each 8-µL hybridization reaction, for a total of 20 µL per reaction.
7. Flick the tube or pipet up and down 5 times to mix, then centrifuge briefly.

8. Incubate the 20-µL ligation reactions in a thermal cycler at 16°C for 2–16 hours.

IMPORTANT! If the starting enriched small RNA is <5 ng, we strongly recommend overnight incubation (16 hours) at 16°C. For a set of experiments, we recommend using the same ligation time for all samples to minimize variation.

IMPORTANT! Set the temperature of the thermal cycler lid to match the block temperature; turn OFF the heated lid; or leave the thermal cycler open during the incubation.

Perform reverse transcription

Required materials from the Ion Total RNA-Seq Kit v2

- Nuclease-Free Water
- 10X RT Buffer
- 2.5 mM dNTP Mix
- Ion RT Primer v2
- 10X SuperScript® III Enzyme Mix

1. On ice, prepare the RT master mix with the ligated RNA sample:

Component	Volume for One Reaction†
Nuclease-Free Water	2 µL
10X RT Buffer	4 µL
2.5 mM dNTP Mix	2 µL
Ion RT Primer v2	8 µL
Total volume	**16 µL**

† Include 5–10% excess volume to compensate for pipetting error when preparing the master mix.

2. Incubate the RT master mix with the ligated RNA sample:
   a. Add 16 µL of RT master mix to each 20-µL ligation reaction.
   b. Pipet up and down 5 times to mix, then centrifuge briefly to collect the liquid in the bottom of the tube.
   c. Incubate in a thermal cycler with a heated lid at 70°C for 10 minutes, then snap-cool on ice.
3. Perform the reverse transcription reaction:
   a. Add 4 µL of 10X SuperScript® III Enzyme Mix to each ligated RNA sample.
   b. Gently vortex to mix thoroughly, then centrifuge briefly.
   c. Incubate in a thermal cycler with a heated lid at 42°C for 30 minutes.

STOPPING POINT The cDNA can be stored at –30°C to –10°C for 2 weeks, stored at –86°C to –68°C for long-term storage, or used immediately.

Purify and size-select the cDNA

Use the Magnetic Bead Cleanup Module twice with the same sample to size-select the desired cDNA products. During the first bead binding, magnetic beads capture larger cDNA species such as tRNA and rRNA. During the second binding and with increased ethanol concentration, desired cDNA products (miRNA and other small RNA) in the supernatant re-bind to the magnetic beads. After washing the beads, the desired cDNA products are eluted with pre-warmed (37°C) Nuclease-Free Water.

Required materials from the Magnetic Bead Cleanup Module

- Wash Solution Concentrate
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate
- Nuclease-Free Water

Other materials and equipment

- 100% ethanol or 200 proof (absolute) ethanol, ACS-grade or higher quality
- Magnetic stand for 96-well plates (Life Technologies, Cat. no. AM10027 or AM10050)
- 37°C heat block or water bath
- (*Optional*) MicroAmp® Clear Adhesive Film (Life Technologies, Cat. no. 4306311)

Before you begin

- If you have not done so already, add 44 mL of 100% ethanol to the Wash Solution Concentrate and mix well. Mark the label on the bottle to indicate that you added ethanol. Store the solution at room temperature (15°C to 30°C).
- If you see a white precipitate in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Nuclease-Free Water at 37°C for ≥5 minutes.

Note: To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp® Clear Adhesive Film (Life Technologies, Cat. no. 4306311). You can also skip a row between sample rows.

IMPORTANT! Accurate pipetting of bead cleanup reagents is critical for best results.

1. Prepare beads for each sample:
    a. Gently vortex the Nucleic Acid Binding Beads tube to completely resuspend the magnetic beads.
    b. Add 7 µL beads to the wells of the Processing Plate.
    c. Add 140 µL Binding Solution Concentrate to each well, then mix the Concentrate and beads by pipetting up and down 10 times.
2. Bind larger cDNA products to the beads:
    a. Transfer each 40-µL RT reaction to a well with beads of the Processing Plate.
    b. Set a P200 pipettor at 120 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the new 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times.

c. Without changing tips, add 120 µL of 100% ethanol to each well.

IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 2b–2c for the remaining wells only if the tip touches the wall Accurate pipetting of 100% ethanol is critical for best results.

---

d. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

Note: The color of the mixture should be homogeneous after mixing.

e. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

3. Remove the supernatant from the beads:

a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

b. Leave the Processing Plate on the magnetic stand, then transfer the supernatant to a new well on the plate or to a well on a new plate.

IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 microliters of supernatant behind.

---

4. Bind desired cDNA products to the beads:

a. Remove the Processing Plate from the magnetic stand.

b. Add 72 µL of Nuclease-Free Water to the supernatant in the new sample well.

c. Set a P100 or P200 pipettor at 78 µL. Attach a new 100-µL or 200-µL tip to the pipettor, then pre-wet the new 100- or 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times.

d. Without changing tips, add 78 µL of 100% ethanol to each well.

IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 4c–4d for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

---

e. Gently vortex the Nucleic Acid Binding Beads tube to completely resuspend the magnetic beads.

f. Add 7 µL beads to the wells of the Processing Plate.

g. Take the samples off of the magnetic stand. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

Note: The color of the mixture should be homogeneous after mixing.

h. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

5. Remove the supernatant from the beads:
    a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
    b. Leave the Processing Plate on the magnetic stand, then carefully aspirate and discard the supernatant from the plate.

    IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 microliters of supernatant behind.

6. Wash the beads with the Wash Solution Concentrate with ethanol:
    a. Leave the Processing Plate on the magnetic stand, then add 150 µL of Wash Solution Concentrate with ethanol to each sample.
    b. Incubate the samples for 30 seconds.
    c. Leave the Processing Plate on the magnetic stand, then aspirate and discard the supernatant from the plate.

    IMPORTANT! Do not disturb the separated magnetic beads. Remove all of the Wash Solution Concentrate from each well.

    d. Use a P10 or P20 pipettor to remove residual ethanol.
    e. Air-dry the beads at room temperature for 1–2 minutes to remove all traces of ethanol.

    IMPORTANT! Do not overdry the beads (overdried beads appear cracked); overdrying significantly decreases elution efficiency.

7. Elute the cDNA from the beads:
    a. Remove the Processing Plate from the magnetic stand.
    b. Add 12 µL of pre-warmed (37°C) Nuclease-Free Water to each sample.
    c. Mix thoroughly by pipetting up and down 10 times.
    d. Incubate the samples at room temperature for 1 minute.
    e. Place the Processing Plate on the magnetic stand for 1 minute to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
    f. For each sample, collect 12 µL of the eluant.

Amplify the cDNA

To prepare non-barcoded libraries, use the following components from the Ion Total RNA-Seq Kit v2:

- Ion 5′ PCR Primer v2
- Ion 3′ PCR Primer v2
- Platinum® PCR SuperMix High Fidelity

To prepare barcoded libraries, plan the barcodes that you want to use, then select the PCR primers from Ion Xpress™ RNA-Seq Barcode 01–16 Kit:

- Ion Xpress™ RNA-Seq Barcode BC 01–BC 16
- Ion Xpress™ RNA 3′ Barcode Primer

1. For each cDNA sample, prepare the PCR mix, according to the preparation of a non-barcoded or barcoded library:

   IMPORTANT! Use the appropriate primers.

Non-barcoded Library	
Component	**Volume for One Reaction†**
Platinum® PCR SuperMix High Fidelity‡	45 µL
Ion 5′ PCR Primer v2	1 µL
Ion 3′ PCR Primer v2	1 µL
Total volume	**47 µL**

† Include 5–10% excess volume to compensate for pipetting error when preparing master mix.

‡ Platinum® PCR SuperMix High Fidelity contains a proofreading enzyme for high-fidelity amplification.

   a. Transfer 6 µL of cDNA sample to a new PCR tube.
   b. Transfer 47 µL of the PCR mix to each 6 µL of cDNA sample.
   c. Proceed to step 2.

Barcoded Library	
Component	**Volume for One Reaction†**
Platinum® PCR SuperMix High Fidelity‡	45 µL
Ion Xpress™ RNA 3′ Barcode Primer	1 µL
Total volume	**46 µL**

† Include 5–10% excess volume to compensate for pipetting error when preparing master mix.

‡ Platinum® PCR SuperMix High Fidelity contains a proofreading enzyme for high-fidelity amplification.

   a. Transfer 6 µL of cDNA sample to a new PCR tube.
   b. Transfer 46 µL of the PCR mix to each 6 µL of cDNA sample.
   c. Add 1 µL of the selected Ion Xpress™ RNA-Seq Barcode BC primer (choose from BC01–BC16) to each PCR tube.
   d. Proceed to step 2.

2. Flick the tube or slowly pipet the solution up and down 5 times to mix well, then centrifuge briefly to collect the liquid in the bottom of the tube.

3. Run the cDNA samples in a thermal cycler:

Stage	Temp	Time
Hold	94°C	2 min
Cycle (2 cycles)	94°C	30 sec
	50°C	30 sec
	68°C	30 sec
Cycle (14 cycles)	94°C	30 sec
	62°C	30 sec
	68°C	30 sec
Hold	68°C	5 min

Purify and size-select the amplified DNA

Use the Magnetic Bead Cleanup Module twice with the same sample to size-select the desired cDNA products. During the first round of bead binding, magnetic beads capture larger cDNA species such as tRNA and rRNA. During the second round of bead binding and with increased ethanol concentration, desired cDNA products (miRNA and other small RNA) in the supernatant re-bind to the magnetic beads. After washing the beads, the desired cDNA products are eluted with pre-warmed (37°C) Nuclease-Free Water.

Required materials from the Magnetic Bead Cleanup Module

- Wash Solution Concentrate
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate
- Nuclease-Free Water

Other materials and equipment

- 100% ethanol or 200 proof (absolute) ethanol, ACS-grade or higher quality
- (*Optional*) Orbital shaker for 96-well plates
- Magnetic stand for 96-well plates (Life Technologies, Cat. no. AM10027 or AM10050)
- 37°C heat block or water bath
- (*Optional*) MicroAmp® Clear Adhesive Film (Life Technologies, Cat. no. 4306311)

Before you begin

- If you have not done so already, add 44 mL of 100% ethanol to the Wash Solution Concentrate and mix well. Mark the label on the bottle to indicate that you added ethanol. Store the solution at room temperature.
- If you see a white precipitate in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Nuclease-Free Water at 37°C for ≥5 minutes.

Note: To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp® Clear Adhesive Film (Life Technologies, Cat. no. 4306311). You can also skip a row between sample rows.

IMPORTANT! Accurate pipetting of bead cleanup reagents is critical for best results.

1. Prepare beads for each sample:
    a. Gently vortex the Nucleic Acid Binding Beads tube to completely resuspend the magnetic beads.
    b. Add 7 µL beads to wells on the Processing Plate.
    c. Add 140 µL Binding Solution Concentrate to each well, then mix the Concentrate and beads by pipetting up and down 10 times.
2. Bind larger amplified DNA to the beads:
    a. Transfer each 53-µL PCR reaction to a well with beads of the Processing Plate.
    b. Set a P200 pipettor at 110 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the new 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times.
    c. Without changing tips, add 110 µL of 100% ethanol to each well.

    IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 2b–2c for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

    d. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

    Note: The color of the mixture should be homogeneous after mixing.

    e. Incubate the samples for 5 minutes at room temperature.
3. Remove the supernatant from the beads:
    a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
    b. Leave the Processing Plate on the magnetic stand, then transfer the supernatant to a new well on the plate or to a well on a new plate.

    IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 microliters of supernatant behind.

4. Bind desired cDNA products to the beads:
    a. Remove the Processing Plate from the magnetic stand.
    b. Add 35 µL of Nuclease-Free Water to the supernatant in the new sample well.

c. Set a P100 or P200 pipettor at 35 µL. Attach a new 100- or 200-µL tip to the pipettor, then pre-wet the new 100- or 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times.

d. Without changing tips, add 35 µL of 100% ethanol to each well.

IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 4c–4d for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results

---

e. Gently vortex the Nucleic Acid Binding Beads tube to completely resuspend the magnetic beads.

f. Add 7 µL beads to the wells of the Processing Plate.

g. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

Note: The color of the mixture should be homogeneous after mixing.

h. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

5. Remove the supernatant from the beads:

a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

b. Leave the Processing Plate on the magnetic stand, then carefully aspirate and discard the supernatant from the plate.

IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 microliters of supernatant behind.

---

6. Wash the beads with Wash Solution Concentrate with ethanol:

a. Leave the Processing Plate on the magnetic stand, then add 150 µL of Wash Solution Concentrate with ethanol to each sample.

b. Incubate the plate at room temperature for 30 seconds.

c. Leave the Processing Plate on the magnetic stand, then aspirate and discard the supernatant from the plate.

d. Use a P10 or P20 pipettor to remove residual ethanol.

IMPORTANT! Do not disturb the separated magnetic beads. Remove all of the Wash Solution Concentrate from each well.

---

e. Air-dry the beads at room temperature for 1–2 minutes to remove all traces of ethanol.

IMPORTANT! Do not overdry the beads (overdried beads appear cracked); overdrying significantly decreases elution efficiency.

---

7. Elute the cDNA from the beads:
   a. Remove the Processing Plate from the magnetic stand.
   b. Add 15 µL of pre-warmed (37°C) Nuclease-Free Water to each sample.
   c. Mix thoroughly by pipetting up and down 10 times.
   d. Incubate the Processing Plate at room temperature for 1 minute.
   e. Place the Processing Plate on the magnetic stand for 1 minute to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
   f. For each sample, collect the 15 µL of eluant.

Assess the yield and size distribution of the amplified DNA

Use the Agilent® 2100 Bioanalyzer® instrument with the Agilent® DNA 1000 Kit.

1. Run 1 µL of the purified DNA on an Agilent® 2100 Bioanalyzer® instrument with the Agilent® DNA 1000 Kit. Follow the manufacturer's instructions for performing the assay.
2. Using the 2100 expert software, perform a smear analysis to determine size distribution of the amplified DNA:
   a. Measure the area for the DNA that is:
      - 50–300 bp (the size range for all of the ligation products)
      - 86–106 bp for non-barcoded libraries or 94–114 bp for barcoded libraries (the size range for the desired miRNA ligation products)
   b. Calculate the ratio of mRNA ligation products in total ligation products using the formula for:
      - Non-barcoded libraries: *[Area (86–106 bp)] ÷ [Area (50–300 bp)]*
      - Barcoded libraries: *[Area (94–114 bp)] ÷ [Area (50–300 bp)]*
   c. Determine the molar concentration of cDNA libraries using size range 50–300 bp. Use this concentration for "Pool barcoded small RNA libraries" and "Determine the library dilution required for template preparation" on page 60.

Note: Adjust the size range to include all library peaks, if necessary.

Note: For instructions on how to perform the smear analysis, see "Perform a smear analysis" on page 68, and refer to the *Agilent® 2100 Bioanalyzer® Expert User's Guide* (Pub. no. G2946-90000).

Next steps

If the ratio is...	Then...
≥50%	Proceed to "Determine the library dilution required for template preparation" or "Pool barcoded small RNA libraries" on page 60.
<50%	Proceed to "Determine the library dilution required for template preparation" on page 60, or "Pool barcoded small RNA libraries" but expect to see an increase in the number of filtered reads (no insert, tRNA, or rRNA mapped reads) when compared to samples with greater than 50% ratio of desired miRNA ligation products to overall products.

Note: Samples that are run on a Bioanalyzer® instrument typically show 5–8 bp larger than their actual size.

Pool barcoded small RNA libraries

Note: If you are not pooling libraries, skip this section and proceed to "Determine the library dilution required for template preparation" on page 60.

1. Determine the molar concentration (nM) of each of the barcoded cDNA libraries with the Agilent® DNA 1000 Kit or the Agilent® High Sensitivity DNA Kit.

   Note: 50–300 bp size range is typically used to determine the library concentration. If necessary, adjust the range to include all of the library peaks.

2. Dilute each barcoded cDNA library to the same molar concentration (nM). For example, if you have 3 different barcoded libraries that are 45, 55, 65 nM, choose a concentration that is equal to or lower than the lowest concentration of the three libraries, such as 30 nM. Dilute all or part of the library to 30 nM.

3. Mix an equal volume of each diluted library to prepare a pool of the barcoded libraries.

4. The final molar concentration of the pooled library is the same for each diluted library. For example, if you dilute each library to 30 nM, the concentration of the pooled library is 30 nM.

   Use the final molar concentration to determine the Template Dilution Factor. You can also determine the molar concentration of the pooled libraries with the Agilent® DNA 1000 Kit or the Agilent® High Sensitivity DNA Kit (see "Assess the yield and size distribution of the amplified DNA" on page 59 and "Using 2100 expert software to assess small RNA libraries" on page 72).

Determine the library dilution required for template preparation

For template preparation using an appropriate Ion template preparation kit, determine the library dilution that gives 210×10^6 molecules of template per 20 µL; 210×10^6 is the recommended library input for Whole Transcriptome libraries. Use a conversion factor of 8.3 nM = 5×10^9 molecules/µL, and the following formula:

Template Dilution Factor =
(Library concentration in nM) × [(5×10^9 molecules/µL)/(8.3 nM)] × [(volume per template preparation reaction inµL)/(210×10^6 molecules)]

For the volume per template preparation reaction, refer to the specific user guide for the appropriate Ion template preparation kit.

If your library concentration is >20 nM, serially dilute 1:10 or 1:100 until your library concentration is ≤20 nM. (This step helps prevent a Template Dilution Factor of several thousands).

Example: The whole transcriptome RNA library concentration is 400 nM. Volume per template preparation reaction is 20 µL.

Dilute 1 µL of library in 99 µL of Nuclease-Free Water (1:100 dilution) to yield a final library concentration of 4 nM. Use this final library concentration to calculate the Template Dilution Factor:

Template Dilution Factor =
(4 nM) × [(5 × 10^9 molecules/µL)/(8.3 nM)] × [20 µL/(210 × 10^6 molecules)] = 229

Thus, 1 µL of the 4-nM library dilution mixed with 228 µL of Nuclease-Free Water (1:229 dilution) yields approximately 210 × 10^6 molecules per 20 µL.

Proceed to template preparation

The library is ready for the template preparation procedure. In this procedure, each library template is clonally amplified on Ion Sphere™ Particles for sequencing on the Ion PGM™ System or Ion PI™ Ion Sphere™ Particles for sequencing on the Ion Proton™ System. For instructions, refer to the specific user guide for an appropriate Ion template preparation kit.

Template preparation documentation is available on the Ion Community at **http://ioncommunity.iontorrent.com/**. Follow the links under **Protocols** > **Prepare Template** > **Prepare Template User Guides and Quick Reference**. You may also refer to the *Preparation and Sequencing of RNA Libraries with the Ion Personal Genome Machine® (PGM™) System User Bulletin* (Pub. no. 4478119).

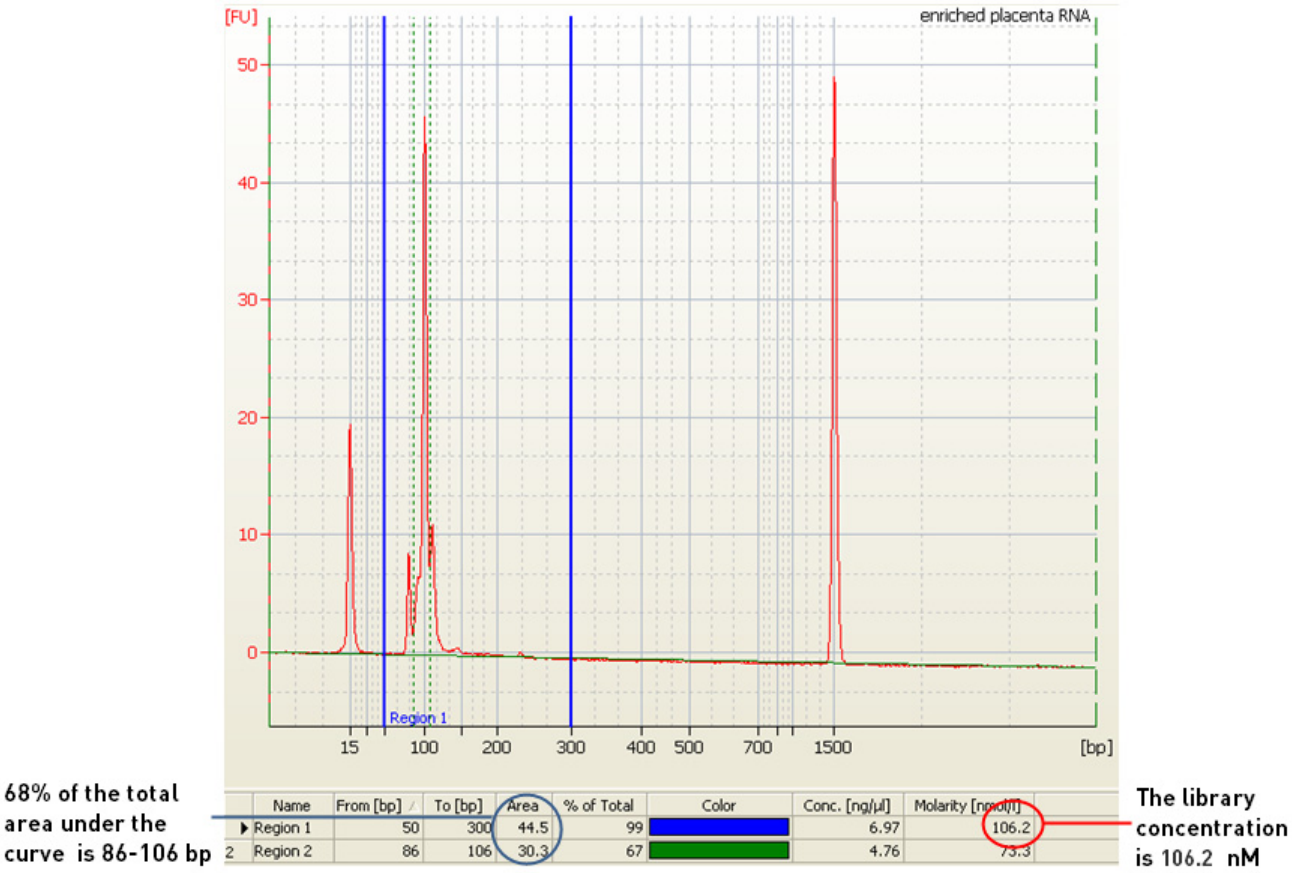
Typical size distribution

Review the plotted and tabulated size distributions in the following sections.

Plotted size distributions

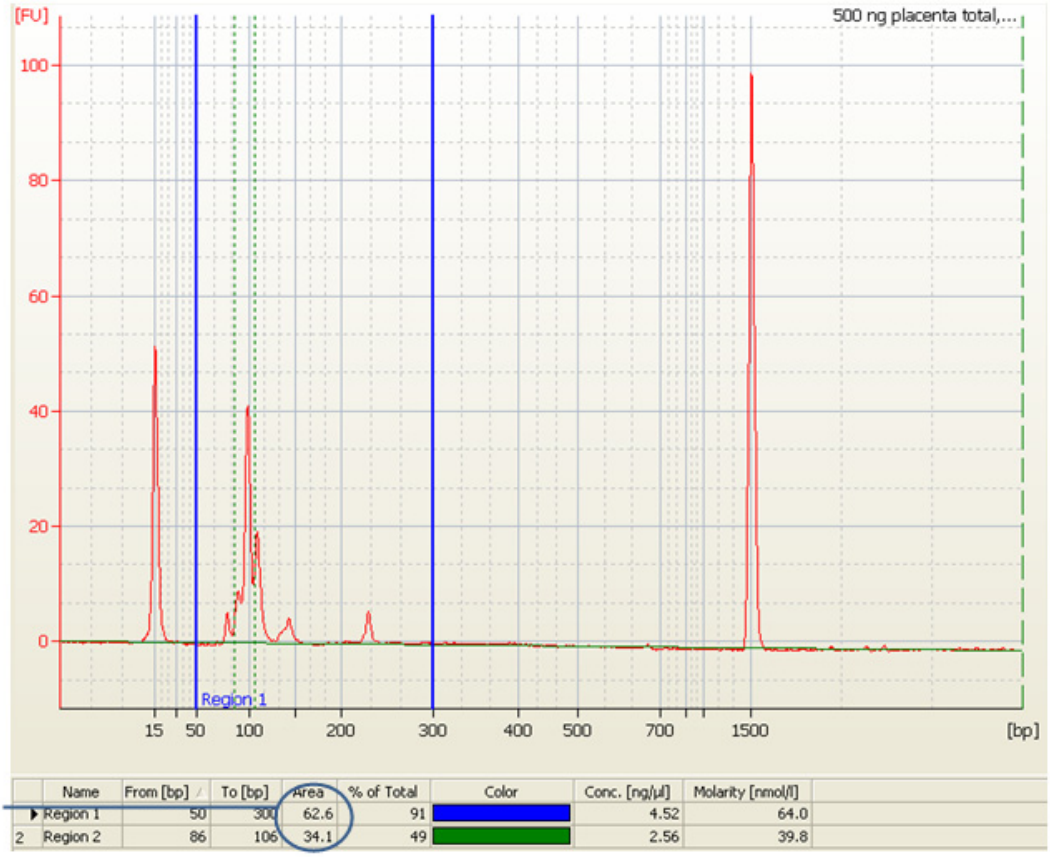
Figure 8 shows the size distribution of non-barcoded, small RNA library from enriched placenta. Figure 9 on page 63 illustrates a typical size distribution of placenta total RNA library (Agilent® 2100 Bioanalyzer® instrument profile). For the highest quality libraries, the ratio of 86–106-bp DNA 50–300-bp DNA is greater than 50%. Figure 10 on page 64 illustrates the size distribution of a barcoded small RNA library prepared from placenta total RNA.

Figure 8 Molar concentration and size distribution of non-barcoded library prepared from enriched placenta small RNA



Note: The amount of tRNA in the final library (reflected by the height of the peak that is about 108 bp on the bioanalyzer trace) varies depending on the lot of placenta you use. Expect to see differences in the ratio of 86–106-bp DNA/50–300-bp DNA when different lots of placenta control RNA are used.

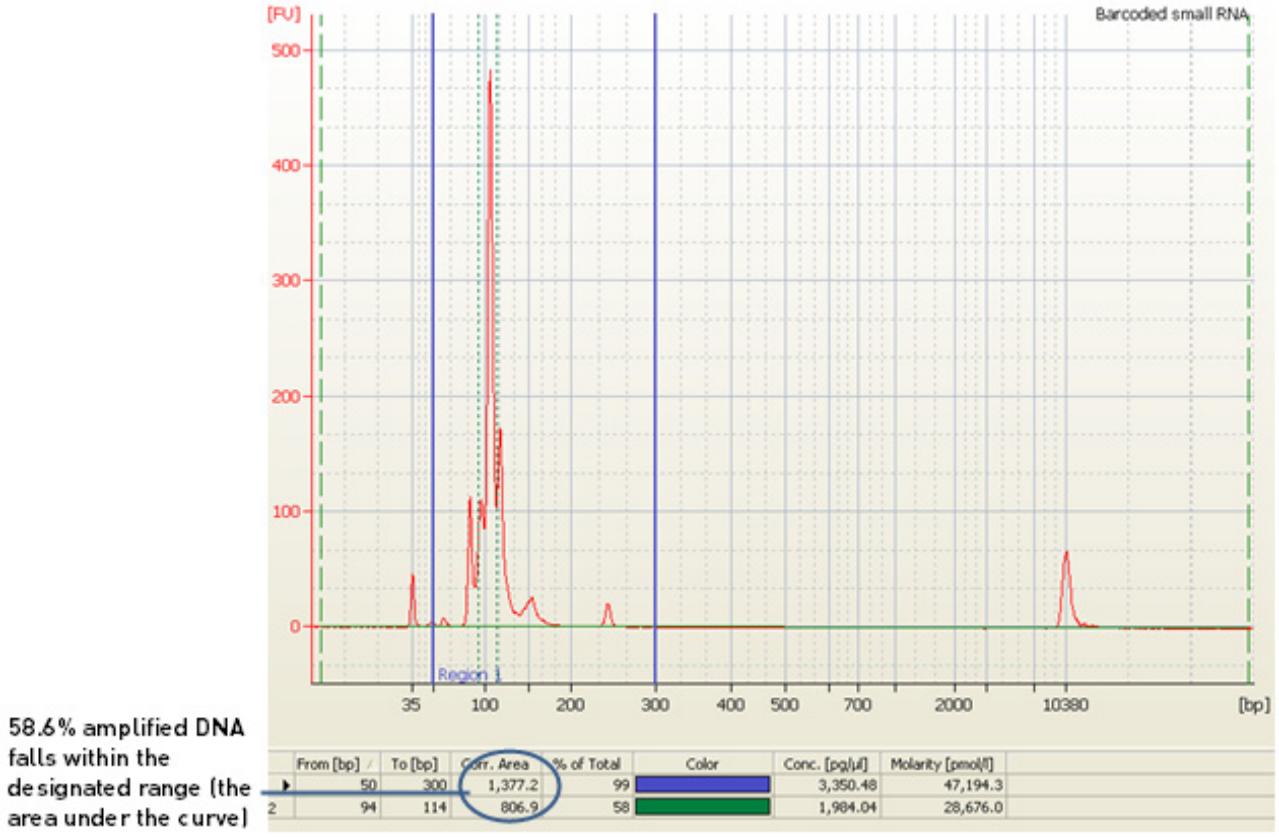
Figure 9 Size distribution of non-barcoded library prepared from placenta total RNA without enriching small RNA



	Name	From [bp]	To [bp]	Area	% of Total	Color	Conc. [ng/µl]	Molarity [nmol/l]
▶	Region 1	50	300	62.6	91		4.52	64.0
2	Region 2	86	106	34.1	49		2.56	39.8

54% amplified DNA falls within the designated range (the area under the curve)

Figure 10 Size distribution of barcoded small RNA library prepared from enriched small RNA



Size distributions compared

Insert Length	Size of *Non-barcoded* Library on the Bioanalyzer® Instrument	Size of *Barcoded* Library on the Bioanalyzer® Instrument
0 bp	~77 bp	~85 bp
10 bp	~87 bp	~95 bp
20 bp	~97 bp	~105 bp
50 bp	~127 bp	~135 bp

Troubleshooting

Observation	Possible Cause	Solution
The Agilent® software does not calculate one concentration and peak size.	The software detects multiple peaks in the amplified cDNA profile.	Refer to "Analyze multiple peaks as one peak" on page 69.
Low yield in the desired size range and high background of smaller <86 bp non-barcoded library or <94 bp for barcoded small RNA library; or >106 bp for non-barcoded library or >114 bp for barcoded library.	Ethanol concentration is incorrect during bead size-selection.	1. Ensure that the ethanol is 100% or 200 proof (absolute). Sub-optimal library size selection with lower ethanol percentage could generate libraries with larger RNA species, such as tRNA and 5S, 5.8S rRNA. 2. Follow the protocol exactly. Some of the steps, such as pre-wetting the tip, are critical for accurate pipetting and correct size selection. 3. Calibrate your pipettor. 4. Using the remaining half of cDNA, repeat PCR and PCR cleanup.
Low yield or only self-ligation products are visible on Bioanalyzer® instrument traces.	Your input amount is too low.	Use enriched or purified small RNA instead of total RNA for ligation or increase the ligation time to 16 hours.
	An enzymatic reaction is not optimal.	Before eluting cDNA from the Nucleic Acid Binding Beads, ensure that the beads are completely dry before adding elution buffer. Residual ethanol can inhibit PCR.
Normal or high yield but PCR products larger than 150 bp.	Too many PCR cycles resulted in overamplification.	Decrease the number of PCR cycles (step 3 on page 56).

Using a positive control to troubleshoot

A general troubleshooting strategy is to perform the Ion Total RNA-Seq Kit v2 procedure using the Small RNA Control (human placenta total RNA) provided with the kit. Use 1 µg of Small RNA Control for the hybridization and ligation procedure starting on page 49.

You can also use the enrichment procedure included in this guide to enrich small RNA from control RNA, then use that as the input for ligation ("Enrich the samples for small RNA" on page 44).

Note: If you are starting from total RNA, the yield will be low; we strongly recommend that you use purified or enriched small RNA.

A Supplemental Information

- Amplified library construction concepts. 67
  - Hybridization and ligation to the Adaptor Mix. 67
  - Reverse transcription and size-selection 67
- Using 2100 expert software to assess whole transcriptome libraries 68
  - Perform a smear analysis. 68
  - Analyze multiple peaks as one peak 69
- Using 2100 expert software to assess small RNA libraries. 72
  - Review the median size 72
  - Perform a smear analysis. 72
  - Determine the % miRNA library 74

Amplified library construction concepts

The procedures in this protocol are based on Life Technologies Ligase-Enhanced Genome Detection (LEGenD™) technology (patent pending).

Hybridization and ligation to the Adaptor Mix

The RNA samples are hybridized and ligated with the Ion Adaptor Mix v2. The Ion Adaptor Mix v2 is a set of oligonucleotides with a single-stranded degenerate sequence at one end and a defined sequence required for the Ion Personal Genome Machine™ (PGM™) System at the other end. The Ion Adaptor Mix v2 constrains the orientation of the RNA in the ligation reaction such that hybridization with the Ion Adaptor Mix v2 yields template for sequencing from the 5′ end of the sense strand. Figure 11 illustrates the downstream emulsion PCR primer alignment and the resulting products of templated sphere preparation for sequencing.

Figure 11 Strand-specific RNA sequence information from Ion Total RNA-Seq Kit products

Reverse transcription and size-selection

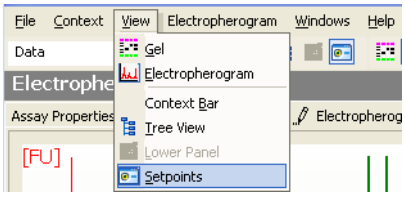
The RNA population with ligated adaptors is reverse transcribed to generate single-stranded cDNA copies of the fragmented RNA molecules. Library generation uses a magnetic bead-based, size-selection process, to enrich for library fragments within the desired size range.

Using 2100 expert software to assess whole transcriptome libraries

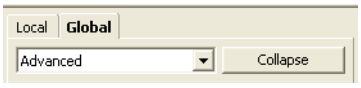
Perform a smear analysis

Perform a smear analysis to quantify the percentage of DNA in the 25–160 bp size range.

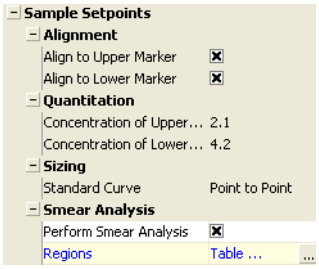
1. In the 2100 expert software, select **View** ▸ **Setpoints**.



2. On the **Global** tab, select **Advanced** settings.

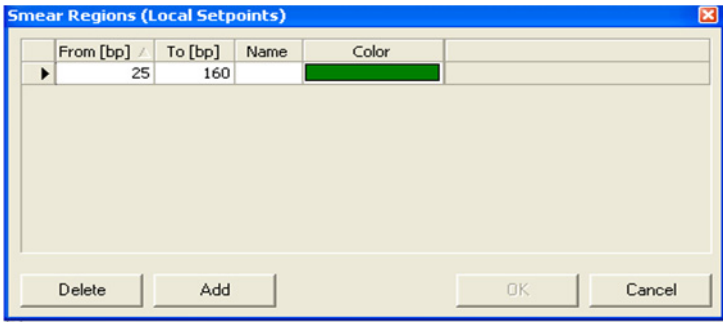


3. In the Sample Setpoints section of the Advanced settings, select the **Perform Smear Analysis** checkbox, then double-click **Table**.



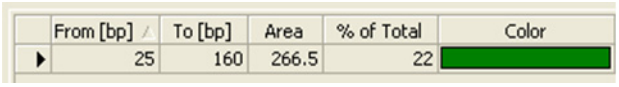
4. Set the smear regions in the Smear Regions dialog box: Click **Add**, then enter **25** bp and **160** bp for the lower and upper limits, respectively.

   These settings are used to determine the percentage of total product that is 25–160 bp in length.



5. Select the **Region Table** tab.

6. In the Region Table, review the percentage of the total product in the size ranges you set.

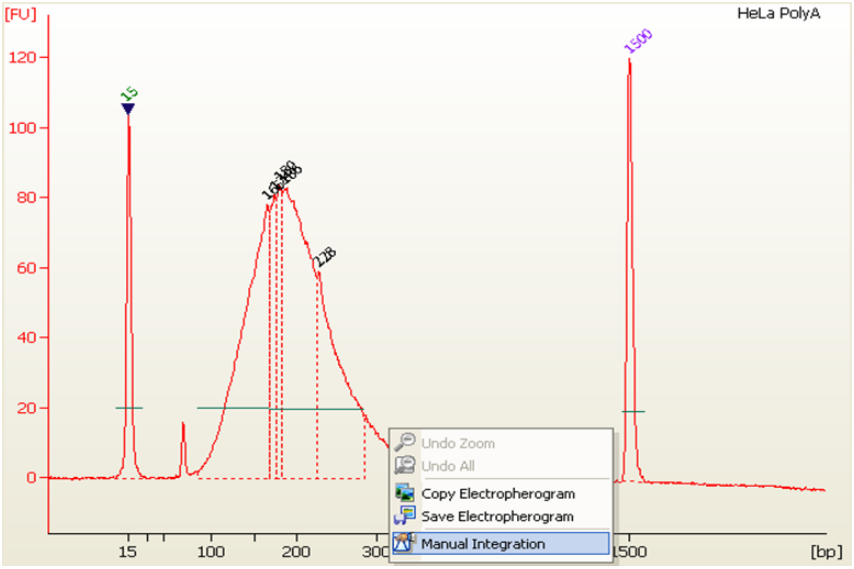


Analyze multiple peaks as one peak

On the Peak Table tab, you may observe that the bioanalyzer software identified multiple peaks in a region that you want to consider as one peak. To obtain one concentration and automatically determine the median size for a peak region, manually set the size range of the desired peak region.

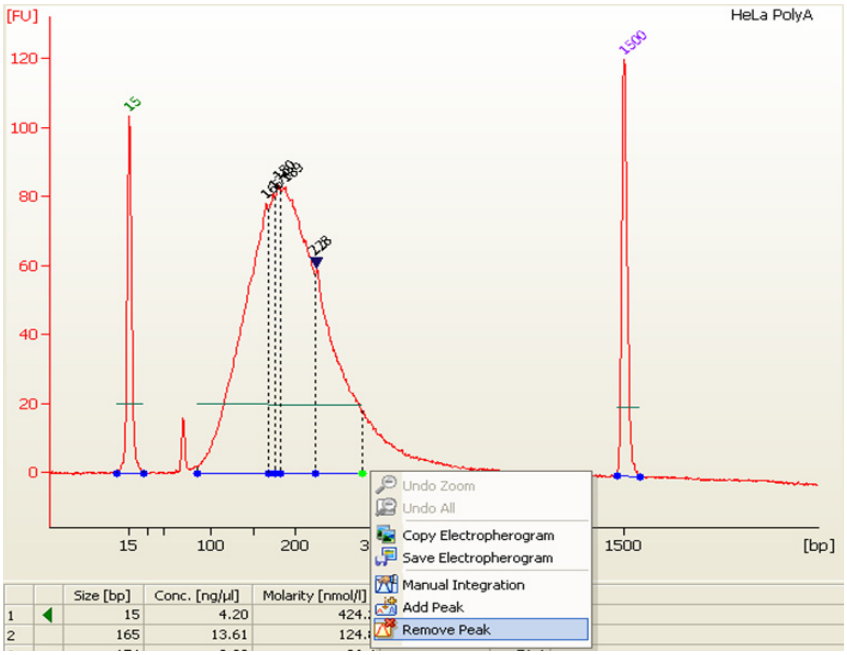
1. In the bottom-left corner of the software window, select the **Peak Table** tab.

2. Right-click anywhere on the electropherogram, then select **Manual Integration**.



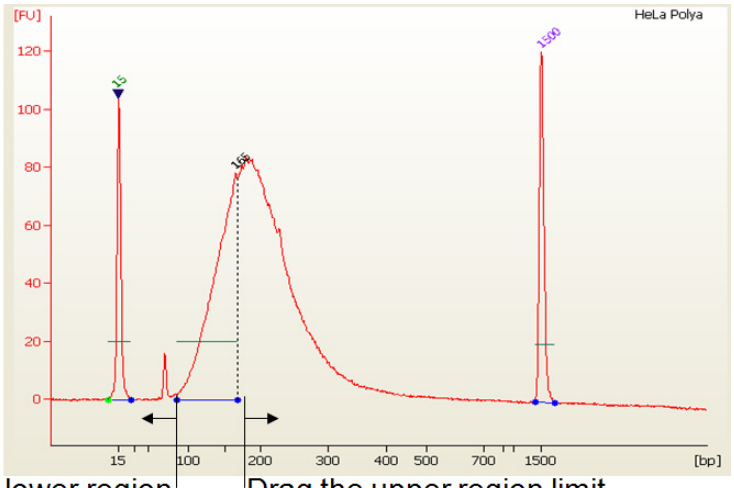
3. To remove multiple peaks:

a. Place the cursor on the peak to remove, right-click, then select **Remove Peak**.

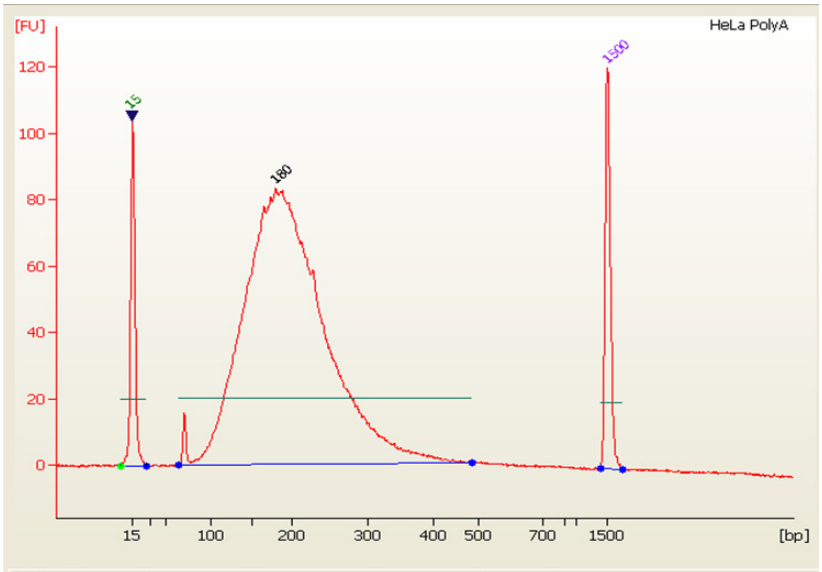


b. Repeat until one peak remains within the region of interest.

c. Drag the lower and upper region limits of the region until the entire library is included.



The software recalculates the median size (bp), concentration (ng/µL), and molarity (nM) of the peak region and displays the values in the Peak Table.



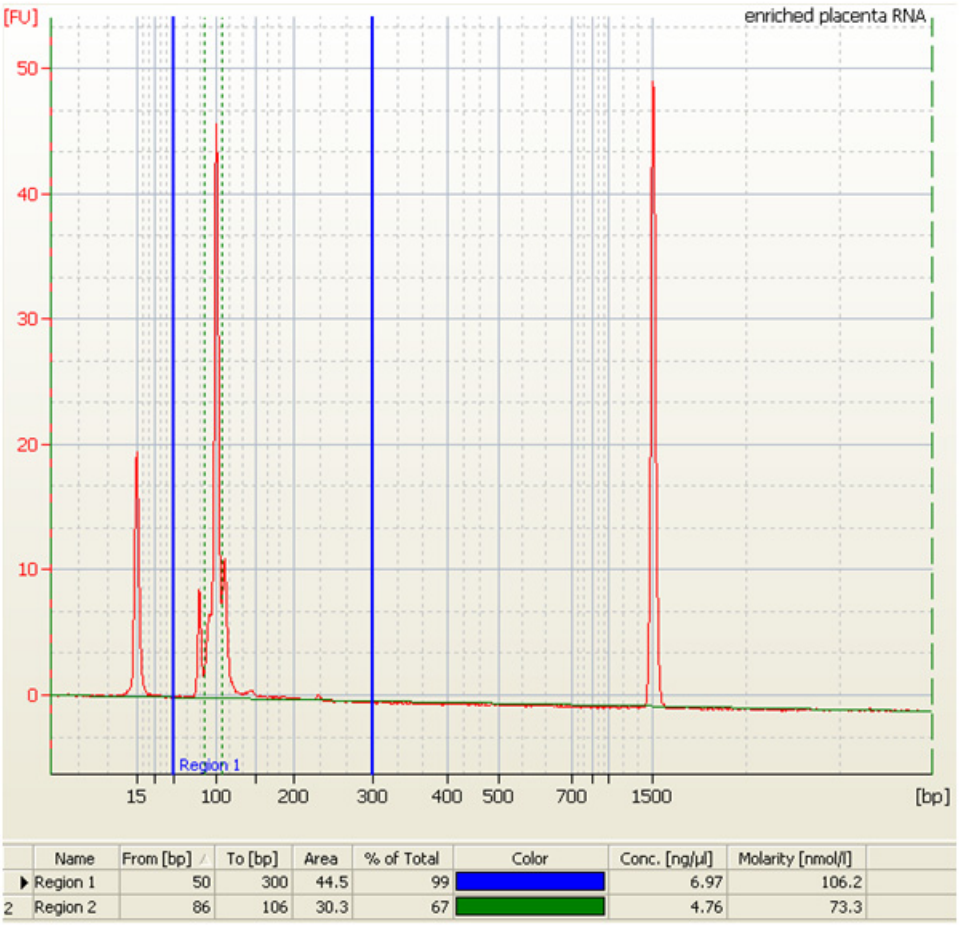
		Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations	Area
1	◀	15	4.20	424.2	Lower Marker	70.5
2		180	43.48	366.7		1,038.2
3	▶	1,500	2.10	2.1	Upper Marker	101.0

Using 2100 expert software to assess small RNA libraries

Review the median size

The 2100 expert software automatically calculates the median size (bp) of miRNA ligation products.

Select the **Peak Table** tab, then review the median size in the Peak Table and at the top of the peak in the electropherogram. The median size should be ~87–91 bp.

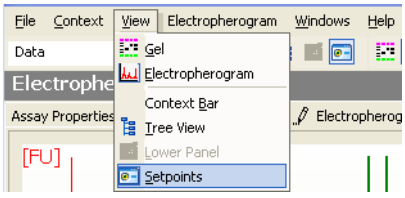


	Name	From [bp]	To [bp]	Area	% of Total	Color	Conc. [ng/µl]	Molarity [nmol/l]
▸	Region 1	50	300	44.5	99		6.97	106.2
2	Region 2	86	106	30.3	67		4.76	73.3

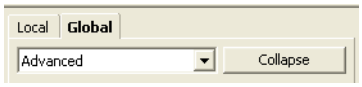
Perform a smear analysis

Perform a smear analysis to quantify the percentage of DNA in the 50–300-bp and 86–106-bp size range. The desired size range for miRNA ligation products is 86–106 bp.

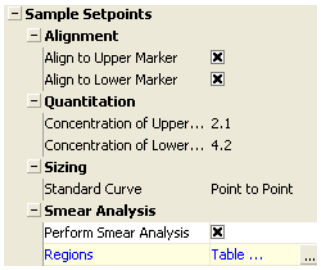
1. In the 2100 expert software, select **View** ▸ **Setpoints**.



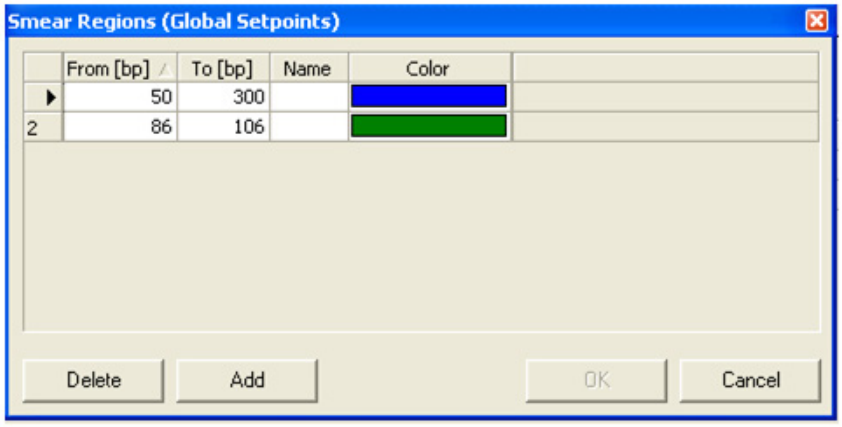
2. On the **Global** tab, select **Advanced** settings.



3. In the Sample Setpoints section of the Advanced settings, select the **Perform Smear Analysis** checkbox, then double-click **Table**.



4. Set the smear regions in the Smear Regions dialog box:
   a. Click **Add**, then enter **50** bp and **300** bp for the lower and upper limits, respectively.
   b. Click **Add**, enter **86** bp and **106** bp, then click **OK**.



5. Select the **Region Table** tab.

6. In the Region Table, review the area values for each of the size ranges you set.

	Name	From [bp]	To [bp]	Area	% of Total	Color	Conc. [ng/µl]	Molarity [nmol/l]
▶	Region 1	50	300	44.5	99		6.97	106.2
2	Region 2	86	106	30.3	67		4.76	73.3

Determine the % miRNA library

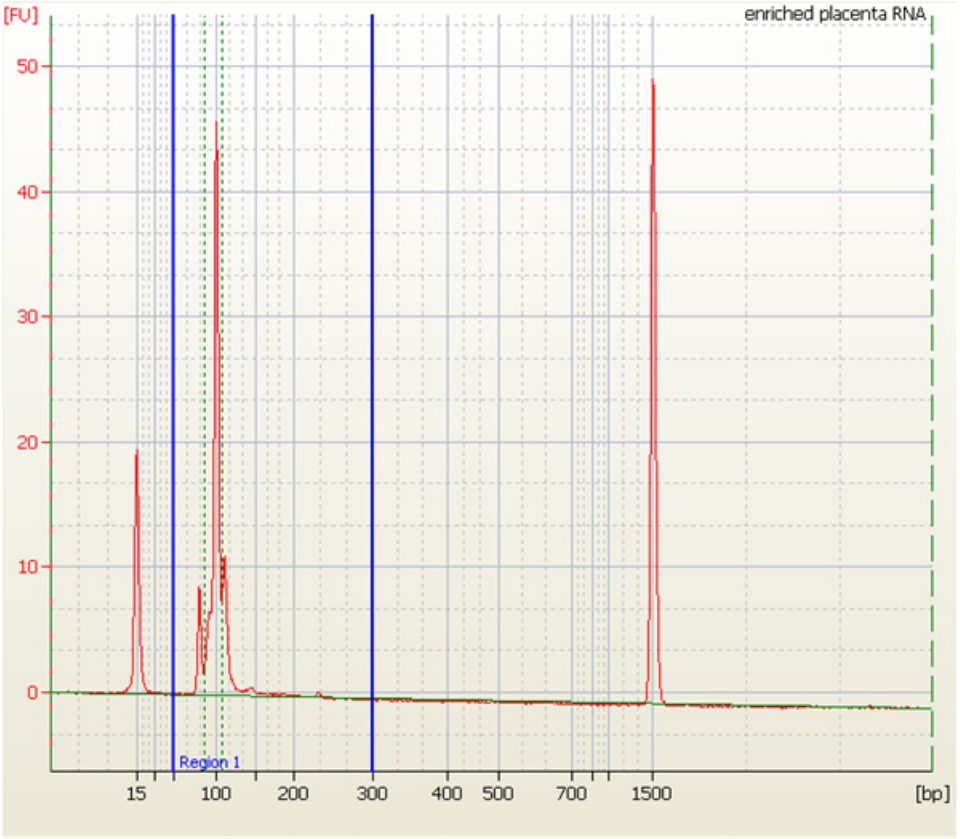
Using the area values from the Region Table, calculate the % miRNA library in the 86–106 bp region as a fraction of the 50–300 bp region using the formula:

% miRNA library = (Area from 86–106 bp ÷ Area from 50–300 bp) × 100

Example % miRNA library calculation

In the example below, the % miRNA library is 68%:

% miRNA library = (30.3 ÷ 44.5) × 100 = 68%



	Name	From [bp]	To [bp]	Area	% of Total	Color	Conc. [ng/µl]	Molarity [nmol/l]
▶	Region 1	50	300	44.5	99		6.97	106.2
2	Region 2	86	106	30.3	67		4.76	73.3

B

Safety

- Chemical safety 76
- Biological hazard safety 77

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc.). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **www.cdc.gov**

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**.

Note: For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtaining Certificates of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

Obtaining support

For the latest services and support information for all locations, go to:

www.iontorrent.com/support

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Ion contact information

Website: www.iontorrent.com

Ion community: ioncommunity.iontorrent.com

Support email: ionsupport@lifetech.com

Phone numbers
In North America: 1-87-SEQUENCE (1-877-378-3623)
Outside of North America: +1-203-458-8552

Addresses

246 Goose Lane Suite 100 Guilford, CT 06437 USA	7000 Shoreline Court Suite 201 South San Francisco, CA 94080 USA

Limited Product Warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288
For support visit lifetechnologies.com/support or email techsupport@lifetech.com

lifetechnologies.com

21 December 2012